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Determination Of The Essential Functions Of A Conserved Cyclin, Cyclin Y, In Drosophila

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DETERMINATION OF THE ESSENTIAL FUNCTIONS OF A CONSERVED CYCLIN, CYCLIN Y, IN DROSOPHILA

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Approved by:

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DEDICATION

献给我的爱人书亮

献给我的父母，公婆，和姐姐们

To my husband, Shuliang, my parents, and sisters, with all my love

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CHAPTER 1

INTRODUCTION

1.1 Summary

Cyclins are a family of highly conserved proteins that activate Cyclin-dependent kinases (Cdks) to regulate the cell cycle, transcription, and other cellular processes. In metazoans, the well-characterized cyclins include CycA, CycB, CycD, and CycE, which play major roles during cell cycle regulation, and CycH, CycT, CycK, and CycC, which are mainly involved in transcriptional regulation. Several additional proteins with the characteristic domain of the cyclin family remain poorly or completely uncharacterized. In Drosophila one of these is encoded by a gene named *CG14939*, which I recently renamed *Cyclin Y* (*CycY*). CycY is a highly conserved protein that has not been functionally characterized in Drosophila, or any other model organism. Only minimal information is available for the human ortholog, CCNY. In this project, I used a reverse genetics approach to characterize the function of Drosophila CycY. I set out to generate and characterize loss-of-function mutants to determine the role of CycY during development. I generated a deletion of the CycY gene (*CycY*) using a technique in which a P-element transposon is mobilized to create an imprecise excision. This deletion, E8, removed the entire *CycY* transcript along with non-coding portions of the adjacent genes. I provided multiple lines of evidence showing that *CycY* is the only gene influenced in E8 and hence renamed this deletion as an allele of *CycY*, $CycY^{E8}$. I also generated transgenic flies that can express dsRNA targeting *CycY* in specific

tissues. The combination of the phenotypic characterization of the *CycY* null mutant and tissue-specific *CycY* knockdown provided initial clues about CycY function in Drosophila. I demonstrated that CycY plays important functions during multiple developmental stages, from embryogenesis to adult viability. The major mutant phenotypes of the zygotic null were visualized during metamorphosis. I also identified the binding partner of CycY, Eip63E (also known as Cdk14), a Cdk induced by ecdysone. Finally I established a connection between CycY and the Brm chromatin-remodeling complex in regulating gene expression. In this chapter, I will introduce background information about cell cycle regulation, CycY, Eip63E and their human orthologs, ecdysone signaling, and Brm complex functions.

1.2 Cell cycle regulation, cyclin-dependant kinases, and cyclins

A number of potentially fatal human diseases like cancer involve cell cycle defects (Hunter and Pines, 1991). Cell cycle control is regulated by an evolutionarily conserved family of serine/threonine protein kinases called cyclin-dependent kinases (Cdks) and their regulatory subunits, the cyclins (Edgar and Lehner, 1996; Morgan, 1995). Critical cell cycle events are both positively and negatively regulated by specific Cdks, whose activities oscillate throughout the cell cycle. Cdk activities are controlled by several different mechanisms, including binding of the positively activating cyclin subunits, inhibition by Cdk inhibitor proteins, phosphorylation by Cdk activating kinases (CAKs) or inhibitory kinases, and dephosphorylation by cell-cycle regulated phosphatases (Petersen et al., 1999).

In the human genome, there are 21 genes encoding Cdks and 5 genes encoding

distantly related proteins known as Cdk-like kinases (CdkLs). These have recently been renamed Cdk1-20 and CdkL1-5 on the basis of similarities in sequence and function, although cyclin partners have not yet been identified for a few of them (Malumbres et al., 2009). Cdks regulate cell cycle progression (Cdk1-4, Cdk6), transcription (Cdk7-10), differentiation (Cdk5), and other cellular processes as well. The activation of a Cdk relies on the binding of a cyclin and on the phosphorylation of specific Cdk residues. For example, Cdk1 by itself is inactive due to a distortion of the ATP-binding site and blockage of the substrate binding by the “T-loop”. The binding of CycB to the highly conserved PSTAIRE helix of the upper kinase lobe causes a conformational change that allows phosphorylation of the tip of the “T-loop” by a Cdk activating kinase (CAK) which is itself a Cdk. This results in movement of the “T-loop” to a group of positively charged amino acids to open the active site. Cyclin binding also activates phosphorylation of the inhibitory site, which holds the Cdk still in an inactive state. Removal of the inhibitory phosphate by the Cdc25 phosphatase finally triggers the activation of Cdk1 (De Bondt et al., 1993; Millar and Russell, 1992). In the absence of a cyclin, the Cdk is inactive regardless of its phosphorylation state. Thus, cyclins are critical regulators of Cdk.

The founding members of the cyclin family, cyclins A and B, were first discovered as proteins that oscillated throughout the cell cycle, peaking in late G2 and M phase (Evans et al., 1983). These proteins were later shown to be required to activate Cdk1, (also known as Cdc2), which is required for entry into M phase in most eukaryotes (Morgan, 1997). At the N terminus of these mitotic cyclins, there is a 9-amino acid destruction motif (RXALG[D/N/E/V]IXN) (D box), which targets the protein for ubiquitin-

dependent degradation by the 26S proteasome during mitosis (Hunt, 1991b). Ubiquitination is mediated by an E3 ubiquitin ligase known as anaphase promoting complex (APC), which has several key targets during mitosis (King et al., 1996). For example, securin, the inhibitor of separase, needs to be degraded at the onset of anaphase to promote sister chromatid separation (Nasmyth, 2002; Nasmyth et al., 2000).

Other cyclins with sequence similarity to cyclins A and B were subsequently identified and shown to be required at other points during the cell cycle (see Table 1-1) (Murray, 2004). The best characterized of these in metazoans include D-type cyclins, which partner with Cdk4 to control G1 phase events, and E-type cyclins, which partner with Cdk2 to control the transition from G1 to S phase. Many of the cellular signals that control entry into S phase function by promoting the stability or degradation of CycD or CycE. D-type cyclins are synthesized in response to growth factor stimulation and persist as long as the stimulation still exists. The protein levels do not show strong oscillation during cell cycle but only form a weak peak near G1-S. Upon serum starvation, they are rapidly degraded (Sherr, 1994). The half-life of these proteins is only about 30 minutes (Hunter and Pines, 1994). The levels of CycE, on the other hand, oscillate with the cell cycle, peaking in late G1. These G1 cyclins do not contain the N-terminal destruction signals found in mitotic cyclins but do contain PEST sequences (enriched for the amino acids Pro, Glu, Ser, Thr) at the C-terminus, which have been suggested to target proteins for rapid degradation (Lew et al., 1991). It was later shown that binding of Cdk2 protected free CycE from degradation by the ubiquitin-proteasome pathway. Phosphorylation of CycE by its cognate Cdk promotes its recognition by

Table 1-1. The regulation of cyclin protein levels during the cell cycle

Cyclin[a] (Drosophila /human)	Oscillation?	Destruction signal	Ubiquitin ligase	References
CycA	Starts to accumulate during S phase and diminishes at mitosis.	destruction box	APC	(Evans et al., 1983; Hunt, 1991a; Hunt, 1991b)
CycB	Synthesized during late S and G2 and degraded at the transition to anaphase.	destruction box	APC	(Evans et al., 1983; Hunt, 1991a; Hunt, 1991b)
CycC	No	No	No	(Loyer et al., 2005)
CycD	Synthesized in response to growth factor stimulation. Forms a weak peak near G1-S.	PEST	unknown	(Hunter and Pines, 1994; Sherr, 1994)
CycE	Peaks at G1/S	PEST	SCF	(Clurman et al., 1996; Koepp et al., 2001; Lanker et al., 1996)
CycF	Starts to accumulate during S phase, peaks in late G2, and declines sharply as cells enter mitosis.	unknown	SCF?[b]	(Tetzlaff et al., 2004)
CycG	Expression induced at G2/M under stress condition.	No	No	(Shimizu et al., 1998)
CycH	No	No	No	(Loyer et al., 2005)
CycJ	Not degraded during mitosis.	No	No	(Althoff et al., 2009)
CycK	No	No	No	(Loyer et al., 2005)
CG16903/CycL	No. Expression induced upon extracellular signal stimulation.	No	No	(Loyer et al., 2005)
CycT	No	No	No	(Loyer et al., 2005)
CycY	Peaks at G2/M.	unknown	unknown	(Davidson et al., 2009)
Koko/FAM58	unknown	unknown	unknown	not available

[a]A generic name for both Drosophila and human cyclins is used in most cases, whereas a species-specific name is used when the gene is not characterized and named as a cyclin in that species. The definition of such proteins as specific cyclins is based on sequence analysis. Drosophila does not have CycF.
[b]CycF is an F-box protein that is part of an SCF complex; therefore, it has been predicted to aid in self degradation.

the protein degradation machinery. The Ser within the PEST sequence is at least one of the phosphorylation sites, which is consistent with the assumption that PEST is part of the destruction signals for G1 cyclins (Clurman et al., 1996; Lanker et al., 1996). This phosphorylation-mediated degradation mechanism provides a self-limiting control of protein level. The ubiquitin ligase responsible for CycE ubiquitination is SCF, which is composed of Skp1, Cul1 (Cdc53), Rbx1, and an F-box containing protein (Koepp et al., 2001).

Several other members of the cyclin family do not show cell-cycle-dependent degradation or synthesis and some have been shown to play roles in cellular processes that are not directly related to cell cycle regulation (Table 1-2). One group of cyclins, for example, regulates transcription by activating Cdks that can phosphorylate the carboxy-terminal tail of the large subunit of RNA polymerase II (Loyer et al., 2005). Several additional members of the cyclin family remain uncharacterized or poorly characterized.

The defining feature of the cyclin family is a homologous region of about 100 amino acids called the cyclin box (Hunt, 1991b), which includes the domain responsible for interaction with a Cdk. Detailed studies on specific Cdk/cyclin complexes have shown that the cyclin box domain is essential and sufficient for interaction with and activation of the Cdk partner (Morgan, 1996). Thus, while specific Cdk partners have not yet been identified for every cyclin, the presence of a cyclin box suggests that all cyclins play the role of activating one or more Cdks. In addition to activating kinase activity, the cyclins may also influence the substrate specificity or determine the subcellular localization of the active complex (Miller and Cross, 2001). Although there are certain specific functions for some cyclins, gene redundancy is also common in the cyclin

Table 1-2. Summary of cyclins, Cdk partners, and the major functions of the cyclin/Cdk complex

Cyclin[a] (Drosophila /human)	Cdk partners	Cellular functions
CycA	Cdk1, Cdk2	CycA/Cdk2 complex is implicated in the control of DNA replication whereas CycA/Cdk1 complex is involved in mitosis by phosphorylating Cdh1 to prevent CycB degradation by APC/Cdh1 (Yam et al., 2002). The S phase role for CycA in Drosophila has also been reported, while in Drosophila, CycA only interacts with Cdk1 (Sprenger et al., 1997).
CycB	Cdk1	CycB/Cdk1 complex is the M phase promoting factor (MPF) in all eukaryotes (Draetta et al., 1989).
CycC	Cdk3, Cdk8	CycC/Cdk3 complex phosphorylates pRB to promote G0-arrested cells to reenter the cell cycle (Ren and Rollins, 2004). CycC also forms a complex with Cdk8 to phosphorylate the C-terminal domain (CTD) of the largest subunit of RNA polymerase II and the CycH subunit of the TFIIH to regulate transcription both positively and negatively (Loyer et al., 2005).
CycD	Cdk4/6	CycD/Cdk4/6 complex promotes G1 progression by phosphorylating pRB (Sherr, 1994). In Drosophila, this complex has been shown to promote cellular growth through mRpL12 and Hif-1 Prolyl Hydroxylase (Hph) (Datar et al., 2000; Frei and Edgar, 2004; Frei et al., 2005; Meyer et al., 2000).
CycE	Cdk2	CycE/Cdk2 complex promotes G1 to S phase transition by phosphorylating pRB, which then releases E2F to activate transcription of genes needed for S phase (Dynlacht et al., 1994; Hinds et al., 1992; Koff et al., 1992).
CycF	no	CycF plays a role in S/G2 that has a subsequent positive impact on G0 cells to reenter cell cycle or G1 to S phase transition. It contains an F-box and assembles into an SCF complex *in vivo*, suggesting it may function in proteolysis (Tetzlaff et al., 2004). It is recently identified to mediate the degradation of CP110, a protein essential for centrosome duplication, and therefore plays a role for mitosis (D' Angiolella et al., 2010).

Table 1-2. Summary of cyclins, Cdk partners, and the major functions of the cyclin/Cdk complex (continued)

Cyclin[a] (Drosophila /human)	Cdk partners	Cellular functions
CycG	Cdk5 (non-Cdk interactors: GAK and PP2A)	CycG associates with Cdk5 and CycG-associated kinase (GAK) (Kanaoka et al., 1997). CycG also associates with PP2A phosphatase to dephosphorylate Mdm2 and hence promotes the degradation of p53 (Okamoto et al., 1996; Okamoto et al., 2002).
CycH	Cdk7/Mat1	CycH/Cdk7/Mat1 complex can function as Cdk activating kinase (CAK) to phosphorylate CycA/B/Cdk1, CycE/Cdk2, and CycD/Cdk4/6. This complex also phosphorylates the CTD of the largest subunit of RNA polymerase II to promote transcriptional elongation as a component of the general transcription factor IIH (TFIIH) (Loyer et al., 2005).
CycJ	Cdk1, Cdk2	CycJ/Cdk2 complex plays a role in the rapid nuclear division cycles of early Drosophila embryogenesis (Kolonin and Finley, 2000) and plays a role in oogenesis (G. Atikukke and R. Finley, unpublished). It has also been reported that CycJ forms a complex with Cdk1 (Althoff et al., 2009).
CycK	Cdk9	CycK/Cdk9 complex phosphorylates CTD of the largest subunit of RNA polymerase II to promote transcriptional elongation (Loyer et al., 2005).
CG16903/CycL	Cdk11	CycL/Cdk11 complex is involved in pre-mRNA splicing (Loyer et al., 2005).
CycT	Cdk9	CycT/Cdk9 complex phosphorylates CTD of the largest subunit of RNA polymerase II to promote transcriptional elongation (Loyer et al., 2005).
CycY	Cdk14	This work.
Koko/FAM58	Cdc2rk[b]	Koko may be required for germline stem cell maintenance (Baker and Kernan, 2007). In human, mutation in FAM58A causes x-linked “STAR syndrome”, while the molecular function is unknown (Unger et al., 2008).

[a]A generic name for both Drosophila and human cyclins is used in most cases, whereas a species-specific name is used when the gene is not characterized and named as a cyclin in that species. The definition of such proteins as specific cyclins is based on sequence analysis. Drosophila does not have CycF.
[b]D. Liu and R. Finley, unpublished data.

family. In Drosophila for example, deletion of either CycA, CycB or CycB3 individually causes no major defects, whereas deletion of both CycB and CycB3, or CycA and CycB3, results in mitotic defects (Jacobs et al., 1998), suggesting substantial overlap among the function of these cyclins. In addition to regulating other downstream substrates, a cyclin can also be the phosphorylation target of its Cdk partner. The phosphorylation may trigger the proteolytic degradation of the cyclin (Ceccarelli and Mann, 2001; Clurman et al., 1996; Lanker et al., 1996) or aid in substrate recognition (Waters et al., 2004).

In summary, a number of proteins in the cyclin family have been identified by virtue of their conserved cyclin box domain (Tables 1-1 and 1-2). Some of these proteins oscillate with the cell cycle like the founding members of the family, while others do not (Table 1-1). Almost all members of the family are thought to partner with specific Cdks. Some cyclin/Cdk complexes regulate the cell cycle, while others regulate transcription or unknown processes (Table 1-2).

1.3 CycY

Several members of the cyclin family, including Drosophila CG14939 (CycY), have not been characterized. In this project, I choose to characterize the biological functions of CG14939 and recently renamed it Cyclin Y (CycY) (Liu and Finley Jr, 2010). CycY is highly conserved in metazoan species. This conservation extends throughout the length of the protein, even outside of the cyclin box, and is more extensive than the conservation of any other type of metazoan cyclin, except for CycC (Liu and Finley Jr, 2010). This level of conservation suggests an important function, yet

prior to last year CycY had not been studied in any organism. Aside from genome sequences, the gene had only been noted in a limited number of functional genomics experiments and genome-wide association studies. A few of these are worth mentioning. A large-scale phosphoproteome study in Drosophila embryos identified several phosphorylated peptides from the *CycY* protein (Zhai et al., 2008). One of the phosphorylation sites, S389, is highly conserved and has also been found to be phosphorylated in human CycY, both in nuclear and cytoplasmic fractions (Beausoleil et al., 2004; Olsen et al., 2006). The human CycY gene, *CCNY*, was identified as a potential susceptibility factor for inflammatory bowel disease (IBD), a complicated genetic disorder affecting the intestinal mucosa. A single nucleotide polymorphism (SNP) located in an intron of *CCNY* was found to be strongly associated with the two IBD subphenotypes, Crohn's disease and ulcerative colitis (Franke et al., 2008; Weersma et al., 2009). Another study found that human CycY is among a number of proteins that are significantly upregulated in metastatic colorectal cancer cells (Ying-Tao et al., 2005). While these results are intriguing, functional studies are needed to determine whether CycY plays any causal role in these diseases.

Recently a handful of independent groups including our own made discoveries about CycY, each from a different starting point (Davidson et al., 2009; Jiang et al., 2009; Li et al., 2009; Liu and Finley Jr, 2010). Combined, these studies have identified a Cdk that is regulated by CycY (Eip63E/PFTK1/Cdk14), found that CycY has a novel plasma membrane localized isoform, demonstrated that it is essential for Drosophila development, and identified a potential role for a CycY-associated kinase in Wnt signaling. These recent findings will be described further in section 1.6 and Chapter 2.

1.4 Eip63E/Cdk14

An initial clue about CycY function came from the fact that Drosophila CycY was isolated through a physical interaction with a Cdk named **e**cdysone-**i**nduced **p**rotein **63E** (Eip63E) in a high throughput yeast two-hybrid (Y2H) screen (Stanyon et al., 2004). The Eip63E gene encodes five highly related and apparently functionally redundant protein isoforms, all of which have homology to cyclin-dependent kinases (Sauer et al., 1996; Stowers et al., 2000). The proteins are most similar to the poorly characterized mammalian Cdk called PFTAIRE, so named because of the amino acid sequence in the conserved helix that binds to cyclins. Although a cyclin partner for Eip63E had not been identified, rescue experiments using mutant variants of the protein have suggested that its activity depends on cyclin binding (Stowers et al., 2000). In those experiments mutation of a conserved glycine adjacent to the PFTAIRE (G243), which in other Cdks is required for cyclin binding, abolished the ability of an *Eip63E* transgene to rescue null mutant embryos to adulthood. Similarly, mutation of a conserved isoleucine (I249), which is also required for cyclin binding in other Cdks, diminished the ability of Eip63E to promote development. A directed yeast two-hybrid screen by Rascle et al., identified two potential regulators of Eip63E, PIF-1B and PIF-2 (PIF stands for **P**FTAIRE **I**nteracting **F**actor), but neither of these proteins has any similarity to cyclins. Both PIF-1B and PIF-2 are cysteine-rich proteins, interacting with the same non-conserved N-terminal histidine-rich domain of Eip63E. It has been proposed that the function of the binding of PIF to the long N-terminal extension of Eip63E, which may inhibit its kinase activity, is to counter the inhibitory effect (Rascle et al., 2003). Subsequent identification

of the Eip63E-CycY interaction in the high throughput yeast two-hybrid screen is not surprising given that Y2H screens are rarely saturating (Schwartz et al., 2009). Furthermore, because Y2H screens can result in false positives, further characterization was needed to show that Eip63E and CycY are functional partners.

The name of Eip63E derives from the fact that one of the three transcription units of the *Eip63E* gene is induced in response to pulses of the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) (Stowers et al., 2000). Ecdysone triggers crucial developmental transitions, including metamorphosis, as described further below. Phenotypic characterization of *Eip63E* loss-of-function mutants has shown that it has essential roles in several developmental processes (Stowers et al., 2000). The majority of zygotic null mutants die during larval development, while only a small percentage survive to pupation. The mutants that survive take 2-3 days longer than their heterozygous siblings to pupariate and are generally smaller than wild type pupae. These phenotypes point to a role for Eip63E in larval development and metamorphosis and further suggest that this Cdk may be involved in growth control. Mutant eye clones, however, showed no morphological or cell cycle defects, leading Stowers et al., to conclude that Eip63E does not regulate the cell cycle (Stowers et al., 2000). *Eip63E* proteins have also been shown to be important for embryogenesis since zygotic null embryos from null mothers fail to hatch into first instar larvae. Interestingly, this maternal effect can be complemented by zygotic expression (Stowers et al., 2000). These findings indicate that this ecdysone-inducible gene is important for metamorphosis and other developmental events as well.

1.5 Ecdysone signaling and metamorphosis

Since *Eip63E* encodes at least one ecdysone induced isoform, and I have shown that CycY plays a role in metamorphosis (Chapter 2) (Liu and Finley Jr, 2010), I will now introduce this important developmental process. Metamorphosis is characterized by striking developmental changes, including the degradation of obsolete larval tissues by programmed cell death and the construction of adult tissues and structures by controlled proliferation, differentiation, and morphogenesis of progenitor cells in the larvae (Riddiford, 1993). The external structures of the adult, such as the head, wing, eye, and leg, develop from larval tissues called imaginal discs or from other primordial cells called histoblasts. The specification of general cell fates (e.g. wing, eye, etc) happens in the embryo, while more specific cell fates are determined during larval development. It is during this stage that imaginal disc cells proliferate rapidly and undergo pattern formation (Cohen, 1993; von Kalm et al., 1995). At metamorphosis, these cells arrest cell division, start differentiation, and initiate the cell shape changes that drive the eversion and elongation to form adult structures (Condic et al., 1991). For example, the wing imaginal disc originates in the embryo within the larval epidermis as a cluster of ~ 50 cells (Bryant and Simpson, 1984; Garcia-Bellido, 1975). These cells are mitotically quiescent before the end of the first larval instar, after which they undergo continuous logarithmic cell proliferation until 24 hr after puparium formation, with an average 8.5 hr cell doubling time (Gonzalez-Gaitan et al., 1994). At the end of the third larval instar, most wing blade cells are arrested in G2 phase and wing margin cells are arrested in G1. These ~ 50,000 wing disc cells are fully grown and patterned to a great degree. After this point, there is still, however, at least one cell division that must occur

during pupal development. During the last 4 hr of the pupal time (20-24 h after puparium formation), there are mitotic events without further DNA synthesis. These cells are finally arrested at G1/G0 phase where they continue differentiation (Milan et al., 1996).

To achieve the final adult structures, cell shapes need to be changed through the contraction of abdominal muscles that drive the eversion and elongation of the imaginal discs (Condic et al., 1991; Fristrom and Fristrom, 1993). For example, failure of the leg imaginal disc to change shape during prepupal development leads to legs not fully extended and/or legs elongated in the wrong direction (D'Avino and Thummel, 1998; Gates and Thummel, 2000). Many factors influence the cell shape changes, such as muscle contraction, rigidity of the pupal cuticle, and proteolysis (Fekete et al., 1975; Fortier et al., 2003). Muscle contraction provides an external force. A less rigid pupal cuticle and the de-attachment of disc cells from the extracellular matrix by protease hydrolysis provide a suitable external environment. Some genes have been reported to be involved in these processes, such as *zip* and *sqh*, encoding subunits of nonmuscle myosin II (Edwards and Kiehart, 1996; Young et al., 1993); *ds*, encoding a member of the cadherin superfamily (Clark et al., 1995); *sb*, encoding a serine protease (Appel et al., 1993); *vlc*, probably functioning at the septate junction; *bl*, encoding a RNA binding protein to regulate transcription or translation (Gates and Thummel, 2000); *crol, E74, BR-C* and *βFTZ-F1*, all of which encode transcription factors induced by ecdysone hormone (D'Avino and Thummel, 1998; Fletcher and Thummel, 1995b; Fortier et al., 2003; Kiss et al., 1988).

The steroid hormone ecdysone is the major insect molting hormone that controls metamorphosis. Ecdysone levels reach six peaks from embryogenesis to pupation

(Richards, 1981a; Richards, 1981b). At the end of third larval instar, the fourth pulse of ecdysone signals puparium formation and the onset of metamorphosis, a set of responses that includes eversion of the imaginal discs to form rudimentary adult appendages, larval tissue histolysis, and the apolysis of the larval cuticle as it forms the puparium (Cohen, 1993). About 12 hours after puparium formation, a brief ecdysone pulse triggers another set of metamorphic responses that includes eversion of the adult head, proliferation of the imaginal histoblast cells to form the epidermis of the adult abdomen, and further histolysis of larval tissues (Bate, 1993; Fristrom and Fristrom, 1993; Skaer, 1993). The final ecdysone pulse lasts throughout the pupation stage and directs differentiation into the adult form. The general mechanism by which ecdysone regulates important transition events during the Drosophila life cycle, especially the larval to pupal transition, has been widely studied. The hormone first binds to a receptor consisting of a heterodimer of ecdysone receptor (EcR) and Ultraspiracle (Usp). Another novel receptor has also been reported (Costantino et al., 2008). The active complex then binds to DNA to induce early or primary response genes, a process that does not require protein synthesis. The protein products from these early genes in turn induce activation of the late genes (secondary response genes). The well characterized primary response genes include *Broad-Complex* (*BR-C*), *E74*, and *E75*, which are all transcription factors (Burtis et al., 1990; Crowley and Meyerowitz, 1984; DiBello et al., 1991; Feigl et al., 1989; Fletcher and Thummel, 1995a; Segraves and Hogness, 1990; Thummel, 1990). Non-transcriptional regulators have also been reported, such as a calcium binding protein encoded by *E63-1*, an ABC transporter encoded by *E23*, and a lipoprotein binding protein encoded by *IMP-E1* (Andres and Thummel, 1995; Hock et al.,

2000; Natzle et al., 1988; Thummel, 1990). Late genes are initially repressed by the ecdysone-receptor complex and are later activated by primary response genes. Traditionally it was assumed that secondary-response genes provide effector functions, which directly affect the metamorphic response. Several well characterized secondary response genes support this assumption (Bayer et al., 1996). The *Sgs* family, for example, encodes glue proteins that are secreted from the salivary gland to help attach the pupae to a dry surface, which protects the animals during metamorphosis (Russell and Ashburner, 1996). *L71* genes encode a series of secreted small polypeptides to provide an antimicrobial defense during metamorphosis (Wright et al., 1996). Other partially characterized secondary response genes, however, may play regulatory roles, such as the Cdk *Eip63E*.

1.6 CycY, Eip63E/PFTK1, and Wg/Wnt signaling

During the past year several independent groups made discoveries about CycY, each from a different starting point (Davidson et al., 2009; Jiang et al., 2009; Li et al., 2009; Liu and Finley Jr, 2010). Here I will introduce some of these new findings.

The human ortholog of Eip63E is PFTAIRE kinase 1 or PFTK1, named for the amino acid sequence in the cyclin-binding domain. The Eip63E/PFTK1 proteins are now known generically as Cdk14 (Malumbres et al., 2009), though the species-specific names will be used here to refer to studies in each organism. Similarly, the human ortholog of Drosophila CycY is CCNY. I will generically call these proteins CycY or use CCNY to refer to studies in human. Little was known about the function of PFTK1. It is expressed in many tissues and is particularly highly expressed in brain, testis, and

ovary in both mouse and human (Besset et al., 1998; Lazzaro and Julien, 1997; Yang and Chen, 2001). A number of PFTK1-interacting proteins have been identified by yeast two-hybrid screens, including CycD3, and more recently CycY (Davidson et al., 2009; Gao et al., 2006a; Gao et al., 2006b; Giot et al., 2003; Jiang et al., 2009; Liu and Finley Jr, 2010; Rascle et al., 2003; Shu et al., 2007; Stanyon et al., 2004). My own work confirmed this interaction for the Drosophila proteins, CycY and Eip63E. Chen and coworkers confirmed the human PFTK1/CycY interaction by co-AP assays from human cells and demonstrated that PFTK1 enhances the serine phosphorylation of CycY, suggesting that CycY may itself be one substrate for the PFTK1/CycY complex. Davidson and coworkers further identified that both Drosophila CycY/Eip63E complex and its human ortholog CCNY/PFTK1 phosphorylate the Wnt co-receptor, known as arrow in Drosophila and LRP6 in humans, and therefore may regulate Wnt signaling (described further below).

One novel feature of CycY is that it spends at least some of its time tethered to the plasma membrane. Jiang et al. showed that human CycY with a C-terminal GFP tag localized to the plasma membrane, a finding confirmed by the Niehrs group (Davidson et al., 2009; Jiang et al., 2009). This localization was dependent on a conserved N-terminal myristoylation signal. Mutation of the putative myristoylation site, glycine 2, to alanine abolished membrane localization, as did fusion to N-terminal tags such as GFP or HA. Jiang et al further showed that forced expression of CycY resulted in relocalization of GFP-PFTK1 from the cytoplasm to the plasma membrane, indicating that this cyclin is capable of recruiting the Cdk to the membrane (Jiang et al., 2009). CycY is the only member of the cyclin family that is known to contain a myristoylation

signal although two non-cyclin proteins that bind Cdk5, p35 and p39, are both myristoylated and require this signal to localize Cdk5 to the plasma membrane (Asada et al., 2008; Humbert et al., 2000; Patrick et al., 1999). The presence of a myristoylation signal and the localization of CycY to the plasma membrane make it likely that CycY functions at least in part by localizing Cdk activity to the plasma membrane to direct phosphorylation of membrane-associated substrates, such as the Wnt co-receptor LRP6/Arrow (Davidson et al., 2009). In cultured cells, myristoylation defective CCNY mutants showed decreased physical interaction with LRP6 and LRP6 S1490 phosphorylation, suggesting the importance of the myristoylation signal and membrane localization for CCNY function, at least for its putative regulatory role of LRP6 (Davidson et al., 2009; Jiang et al., 2009). The human *CCNY* gene and one of its paralogs (*CCNY-like 1*) each encode two splice isoforms, which differ in the presence or absence of the N-terminal 54 amino acids containing the N-terminal myristoylation signal. The shorter splice variant (isoform 2, originally named CycX) of human CycY was cloned and shown to be mostly nuclear (Li et al., 2009). Thus, it is possible that a form of CycY functions in the cytoplasm or nucleus to promote phosphorylation of substrates other than LRP6.

In cultured cells, CCNY level oscillates throughout the cell cycle, peaking at G2/M. The protein also appears to be subject to ubiquitination mediated protein degradation (Davidson et al., 2009). PFTK1 also peaks at G2/M, coincident with the maximal LRP6 phosphorylation and Wnt signaling. The induction of Wnt signaling at G2/M by CCNY/PFTK1 phosphorylation has been proposed to orchestrate a mitotic program (Davidson et al., 2009). Besides CCNY, PFTK1 has also been shown to be activated by another cyclin, CCND3, and inhibited by $p21^{Cip1}$, and to phosphorylate Rb.

Ectopic expression of *PFTK1* in U2OS cells specifically promotes G1 to S transition, and *PFTK1* knockdown in SH-SY5Y cells arrests cells at G1 phase (Shu et al., 2007). Consistently, in Drosophila S2 cells, *Eip63E* knockdown led to decreased mitotic index (Bettencourt-Dias et al., 2004). These cell cycle related phenotypes may be a synergistic effect from both LRP6 and Rb phosphorylation involving CCNY and CCND3 respectively. It is still unclear why a Cdk that is enriched at G2/M has major cell cycle related effects during the transition from G1 to S phase. Whether there are other cyclins or substrates that also contribute to the PFTK1-related cell cycle regulation requires further investigation.

The Wnt/Wingless (Wg) signaling pathway is conserved during evolution. All the components in the mammalian Wnt signaling have counterparts in the Drosophila Wg signaling pathway (Table 1-3). The pathway is required for pattern formation during embryonic development, cell proliferation, differentiation, and other biological processes. Wnt are secreted signaling molecules involved in multiple signaling pathways, one of which is the canonical Wnt/β-catenin cascade. In the absence of Wnt, the transcription factor β-catenin is phosphorylated at serine 45 by CK1, which is recruited by the scaffold protein Axin. This phosphorylation primes the subsequent phosphorylation by GSK3, another Axin binding protein, at β-catenin serine 41, 37, and 33. The fully phosphorylated β-catenin is recognized and ubiquitinated by the β-TrCP E3 ubiquitin–ligase complex, and transferred by the adenomatous polyposis coli (APC) tumor suppressor gene product, a third Axin binding protein, from the Axin complex to the 26S proteasome to be degraded. The Dishevelled (Dvl) protein is also capable of interacting with Axin and inhibiting the Axin-mediated GSK3-dependent phosphorylation

Table 1-3. Wnt/Wg pathway components

Mouse/Human	Drosophila	Function
Wnt	Wingless (Wg)	Secreted ligand
Frizzled (Fz)	Frizzled (Fz)	Wnt/Wg receptor
LRP5/6	Arrow (Arr)	Wnt/Wg co-receptor
Dishevelled (Dvl)	Dishevelled (Dsh)	Scaffold protein
GSK3	Shaggy/Zeste-white 3 (Sgg)	LRP6/Arr kinase
CK1	Gilgamesh (Gish)	LRP6/Arr kinase
Axin	Axin	Scaffold protein
APC	APC	Scaffold protein, tumor suppressor
β-catenin (β-cat)	Armadillo (Arm)	Transcription factor
Tcf/Lef	dTcf/Pangolin (Pan)	Transcription factor

and degradation of β-catenin (Kishida et al., 1999). Upon Wnt stimulation, the single pass transmembrane Wnt co-receptor LRP6 aggregates and recruits Dvl, GSK3, and Axin to the cell membrane to assemble a complex called the LRP6 signalosome. In the signalosome, the intracellular domain of LRP6 is phosphorylated by GSK3 on PPPSP motif and by CK1γ (a membrane-anchored member of the CK1 family) on other sites including T1479. This removes the autoinhibitory effect exerted by the LRP6 extracellular domain on itself. These sequential translocation and phosphorylation events finally release β-catenin from the Axin degradation complex. The free cytoplasmic β-catenin then translocates to the nucleus and binds Tcf/Lef to regulate the transcription of Wnt target genes (Verheyen and Gottardi, 2010).

In addition to the ligand-dependent phosphorylation of LRP6 by GSK3 and CK1γ, it had been noted that the LRP6 receptor also undergoes a ligand-independent phosphorylation at residue S1490, which may be required for or enhance the subsequent ligand-dependent phosphorylations (Davidson et al., 2005). To search for the kinase(s) responsible for ligand-independent S1490 phosphorylation, the group performed a screen in which they individually knocked down the expression of every kinase by RNAi in Drosophila S2R+ cells and screened for decreases in both Wnt signaling and LRP6 S1490 phosphorylation. The investigators identified Eip63E and were led to its potential partner (CycY) by the previously mentioned yeast two-hybrid data. Kinase assays using immunopurified Eip63E and CycY showed that in combination they significantly enhanced phosphorylation of LRP6. Using RNAi knockdown in Drosophila and human cells they were able to show that both Eip63E and CycY are required for maximal LRP6 S1490 phosphorylation and for maximal Wnt

signaling. These results are consistent with a model in which membrane-associated CycY recruits Cdk14 to the membrane where it phosphorylates LRP6 to help prime LRP6 for activation by Wnt. Although membrane localization has not yet been demonstrated for Drosophila CycY, it does have the N-terminal myristoylation signal and the signal is required for LRP6 S1490 phosphorylation in cultured cells (Davidson et al., 2009).

1.7 Brm ATP-dependent chromatin-remodeling complex function

CycY has been identified to physically interact with Snr1 in a high throughput yeast two-hybrid screen (Giot et al., 2003), which prompted us to investigate the relationship between CycY and Snr1, and to test the possibility that Snr1 is a downstream target of CycY/Eip63E complex.

Snr1 (**SN**F5-**r**elated **1**) is the Drosophila counterpart of yeast SNF5 and human hSNF5/INI1 (**In**tegrase-**i**nteracting protein **1**). It is one of the core subunits of the SWI/SNF ATP-dependent chromatin-remodeling complex, which in Drosophila is known as the Brahma (Brm) complex (Dingwall et al., 1995). Chromatin is mainly composed of DNA and histone proteins. In order to fit the relatively long DNA into a small nucleus while maintaining appropriate access to it, DNA is wound around a histone protein core, which together forms a highly compact structure. During the processes of DNA replication, recombination, repair and transcription, the chromatin has to be remodeled to make DNA accessible. There are four classes of ATP-dependent chromatin remodelers, SWI/SNF, ISWI, Mi-2, and Ino80, each of which has a unique ATPase and subunit composition (Mohrmann and Verrijzer, 2005). All of these use the energy from

ATP hydrolysis to modulate the relative position of DNA and histones. The ATPase in the SWI/SNF complex is SWI2/SNF2 or Sth1 in yeast, Brm in Drosophila, and hBrm or Brg1 in human. By modulating chromatin structures, SWI/SNF complex is involved in both gene activation and suppression. In yeast, about 5-7% of genes are regulated by SWI/SNF complex activity based on microarray studies in mutants (Monahan et al., 2008; Sudarsanam et al., 2000)s. In Drosophila, about 2% of the genes are differentially regulated in Brm complex mutants (Zraly et al., 2006). The genes regulated by the SWI/SNF complex in metazoans include genes required for cell proliferation and several components of the Brm complex have been suggested to be tumor suppressors, including hSNF5, Brg1, and Brm (Reisman et al., 2009). One important mechanism by which SWI/SNF complex activity is regulated is phosphorylation. For example, Brg1 and BAF155 both have been found to be phosphorylated by the CycE/Cdk2 complex and the expression of CycE can overcome the ability of Brg1 to induce growth arrest (Shanahan et al., 1999).

1.8 Project outline

The goal of the studies described in this dissertation was to characterize the function of a novel conserved cyclin, CG14939 (CycY) in Drosophila. In Chapter 2, I describe the generation of a null mutant allele of *CycY* and show that its phenotype is similar to that of *Eip63E* mutants. I show that CycY plays major essential roles during metamorphosis, especially during pupariation. I also show that maternally provided CycY is essential for embryogenesis and that this requirement could be partially rescued by zygotic expression. Finally, I confirm that CycY and Eip63E specifically

interact and show that the interaction depends on a conserved phosphorylation target on CycY, Ser389. This part of work has been published recently (Liu and Finley Jr, 2010).

In Chapter 3, I describe the generation of transgenic flies for knocking down the expression of *CycY* in specific tissues. By combining data from the conditional knockdown and the null mutant allele, I show that CycY is required for wing growth and sustained adult viability. I also show that CycY genetically interacts with two components of the Brm ATP-dependent chromatin-remodeling complex, Snr1 and Brm, and that CycY and Snr1 can physically interact. Moreover, the downstream targets of Brm complex, *Eig71Eh* and *Eig71Ei*, are misregulated in *CycY* mutants. Taken together, these data suggest that CycY may be involved in gene regulation through modulating the Brm complex activity.

Data presented in this dissertation have provided initial interesting findings about essential *in vivo* requirements for CycY. Perhaps more importantly, the establishment of the *CycY* null mutant strain and a variety of transgenic lines opens the door for further studies. In Chapter 4, I present the results of several additional experiments aimed at a better understanding of CycY function. In Chapter 5, I summarize the important conclusions from this study and discuss several interesting questions that it raised. I also propose future experiments to address some of the questions.

CHAPTER 2

CYCLIN Y IS A NOVEL CONSERVED CYCLIN ESSENTIAL FOR DEVELOPMENT IN DROSOPHILA

Part of the work described in this chapter has been published in ***Genetics*** **184: 1025-1035, 2010.**

2.1 INTRODUCTION

Cyclins are a superfamily of eukaryotic proteins that play a critical role in activating a group of S/T kinases called cyclin-dependent kinases (Cdks). Well studied metazoan cyclins have two major biological functions, regulating the cell cycle (CycA, CycB, CycD, CycE) and transcriptional regulation through RNA pol II (CycH, CycT, CycK, CycC). CG14939 (CycY) is one of the few poorly or fully uncharacterized cyclins in Drosophila. Alignment of the amino acid sequences of all cyclins from one species shows that there are two sequence classes that roughly correspond to the two major functional categories. CycY, however, does not fit neatly into either one of these two sequence classes. The sequence alone, therefore, has not provided any clues to CycY's cellular functions. One powerful approach to elucidate the *in vivo* biological functions of a gene is to create loss-of-function mutants and examine their phenotypes. Here I set out to do this for *CycY*.

In Drosophila, a number of *in vivo* mutagenesis techniques are available, including gene targeting by homologous recombination (Rong and Golic, 2000; Rong et al., 2002), creation of a deletion by FRT-mediated recombination (Golic and Golic, 1996; Parks et al., 2004), P-element-mediated imprecise excision (Robertson et al.,

1988), and conditional knockdown by using Gal4/UAS-RNAi systems (Lee and Carthew, 2003). Gene targeting by homologous recombination is theoretically a great way to create specific mutations in the gene of interest (Rong and Golic, 2000; Rong et al., 2002). However, due to uncertain technical issues, reports of successful mutant generation with this approach have been rare. Insertional mutagenesis using transposons is a useful gene disruption technique that is now being applied on a genome-wide basis in Drosophila (Bellen et al., 2004; Thibault et al., 2004), increasing the likelihood that a locus of interest will have a P-element in it or nearby. However, in many cases, the insertion itself will not cause any detrimental effect on the function of a gene. Looking through all available P-element or PiggyBac transposon insertion lines around the *CycY* genomic region, I found only one P-element insertion line, d03228, which is from the Exelixis mutant collection (Thibault et al., 2004). This P-element is inserted in between the *CycY* stop codon and the start codon of its neighboring gene *crol*. Adult flies bearing this insertion do not show any visible developmental defects, suggesting that the genes around this insertion site are still functional. However, this is a good candidate strain to be used for P-element imprecise excision. In this approach, the P-element is mobilized by transiently expressing transposase. At some frequency the P-element will be excised imprecisely, resulting in deletion of nearby sequences. The gene arrangement around the *CycY* region on the chromosome is very simple. There is no overlap between neighboring genes and they are not particularly close to each other. This should enable the creation of a *CycY* null mutant that has the neighboring genes intact. Finally, knocking down gene expression with dsRNA is

becoming a widely used and efficient approach, although the caveat of off-target-effects always exists. This approach will be discussed further in Chapter 3.

In this chapter, I successfully generated a null mutant of *CycY* using the imprecise excision approach. I further show that the null mutant, $CycY^{E8}$, is homozygous lethal with most mutant animals arresting during pupal development. The mutant exhibits delayed larval growth and major developmental defects during metamorphosis, including impaired gas bubble translocation, head eversion, leg elongation, and adult tissue growth. Heat shock-induced expression of *CycY* at different times during development resulted in variable levels of rescue, the timing of which suggests a key function for zygotic *CycY* during the transition from third instar larvae to prepupae. *CycY* also plays an essential role during embryogenesis since zygotic null embryos from null mothers generated with germline clones fail to hatch into first instar larvae. I provide evidence that the *CycY* protein (CycY) interacts with Eip63E, a Cyclin-dependent kinase (Cdk) for which no cyclin partner had previously been identified. Like *CycY*, the *Eip63E* gene has essential functions during embryogenesis, larval development, and metamorphosis. Our data suggest that CycY and Eip63E form a cyclin/Cdk complex that is essential for several developmental processes.

2.2 MATERIALS AND METHODS

2.2.1 Fly stocks

All fly stocks were maintained in vials containing standard cornmeal molasses medium and raised at 25°C unless otherwise stated. Fly strains used in this study are listed in Appendix A.

2.2.2 Plasmid cloning for P-element transformation and expression in Drosophila tissue culture cells

pAS1 (A. Soans, and R.L.F., unpublished) is a modified pUAST (Brand and Perrimon, 1993) vector encoding a myc-tag followed by 5' and 3' recombination tags (RTs) to facilitate cloning of open reading frames containing the same RTs from yeast two hybrid vectors (Giot et al., 2003; Stanyon et al., 2004). A map of pAS1 is available at http://www.proteome.wayne.edu. pAS1-CycY was constructed by subcloning a fragment of the *CycY* cDNA beginning with the ATG and ending with the stop codon. The fragment was generated by PCR from the yeast two-hybrid clone using oligonucleotides [Forward: 5' TTGACTGTATCGCCGGAATTC (5'RT-F, Finley lab # 891); Reverse: 5' CCGGAATTAGCTTGGCTGCAG (3'RT-R, Finley lab # 892)], which provided the 5' and 3' RTs at either end, respectively. The fragment was subcloned by gap repair in *E.coli* (Parrish et al., 2004). The P{*CycY*} genomic clone was constructed by sequentially subcloning a 3.6 kb BamHI/NotI fragment and then a 4.0 kb AvrII/EcoRI fragment, each from BACR05B13 (BACPAC resources center), into pCaSpeR2 (Thummel et al., 1988). The whole insert is 7.3 kb, which includes the entire *CycY* gene

and sequences 4,032 bp upstream of the *CycY* start codon, and 1,970 bp downstream of the stop codon, and includes none of the coding regions of *crol* or *Pde1c*. A P-element carrying the *CycY* cDNA expressed from a heat shock promoter, pCaSpeR-hs-CycY, was constructed by subcloning the 1.5 kb HpaI/StuI fragment encoding myc-CycY from pAS1-CycY into the HpaI/StuI sites of pCaSpeR-hs (Thummel et al., 1988). P-element mediated transformation was performed as previously described (Rubin and Spradling, 1982).

pDL4 is a derivative of pUAST-NTAP (Veraksa et al., 2005) containing the NTAP tag followed by the same 5' and 3' RTs found in pAS1. pDL2 is modified from pAS1 by replacing the myc tag with GST tag. pDL5 is modified from pAS1 and pCeMM-NTAP (GS) by replacing the myc tag with NTAP (GS) tag (Burckstummer et al., 2006). This NTAP tag (GS) is composed of two IgG binding domains of Staphylococcus aureus protein G and a streptavidin-binding peptide, separated by a TEV protease cleavage site. Coding regions from the ATG to the stop codon of various Cdks, cyclins, and other proteins were amplified from yeast two-hybrid clones (Stanyon et al., 2004) with primers that added the 5' and 3' RTs and the products were subcloned into pAS1, pDL4, pDL5, or pDL2 by gap repair in *E.coli*. Plasmids used or constructed in this study are listed in Appendix B.

2.2.3 Establish stable transgenic fly strains

All the fly embryo microinjections for P-element-mediated transformation were performed by Duke University. We received from Duke second or third instar larvae that developed from microinjected embryos. A few drops of sterile water were added to the

food surface upon receiving these vials. Each individual G0 male and female was crossed with w^{1118} females and males respectively. Red-eyed G1 progeny were crossed with a double balancer strain, Finley lab # 41 (*w*; CyO/Sp; TM3 Ser/Sb*). Red-eyed F1 progeny with *CyO* and *TM3 Ser* balancer chromosomes were crossed again with the double balancer strain Finley lab # 41. For each F1 transgenic fly, a stable line was established by collecting the progeny with *CyO* and *Ser* balancer chromosomes and at least one marker chromosome (*Sp* or *Sb*). The genotype of the transgenic flies with transgene on the second chromosome is *w*; CyO/transgene; TM3 Ser/Sb*. The genotype of the transgenic flies with transgene on the third chromosome is *w*; CyO/Sp; TM3 Ser/transgene*. Any progeny with four markers, *CyO*, *Sp*, *Ser*, and *Sb*, suggested that the transgene in this line was probably located on the X chromosome. In this case, either a self-cross of the red-eyed progeny with all four markers was done to generate stable homozygous lines, or a cross with an X chromosome balancer strain Finley lab # 270 ($FM7i/Rok^2$). All transgenic lines generated for this work are listed in Appendix C.

2.2.4 Generation and molecular characterization of a *CycY* mutant allele

I used P-element imprecise excision to generate *CycY* mutant alleles. The starting P element in d03228 is inserted 1,958 bp downstream of the *CycY* stop codon and 5,723 bp upstream of the *crol* start codon (Figure 2-1). The genetic crossing scheme is shown in Figure 2-2. d03228 virgin females were mated with *w**; *CyO*/*Sp*; *Δ2-3*, *Sb*/*TM6* males, which provided P-transposase. F1 females (*w*; d03228/CyO; Δ2-3, Sb/+*) were then mated to *w**; *CyO*/*Sp*; TM3, *Ser*/*Sb* males, and F2 progeny were screened for P-element excision by the reversion of eye color to white

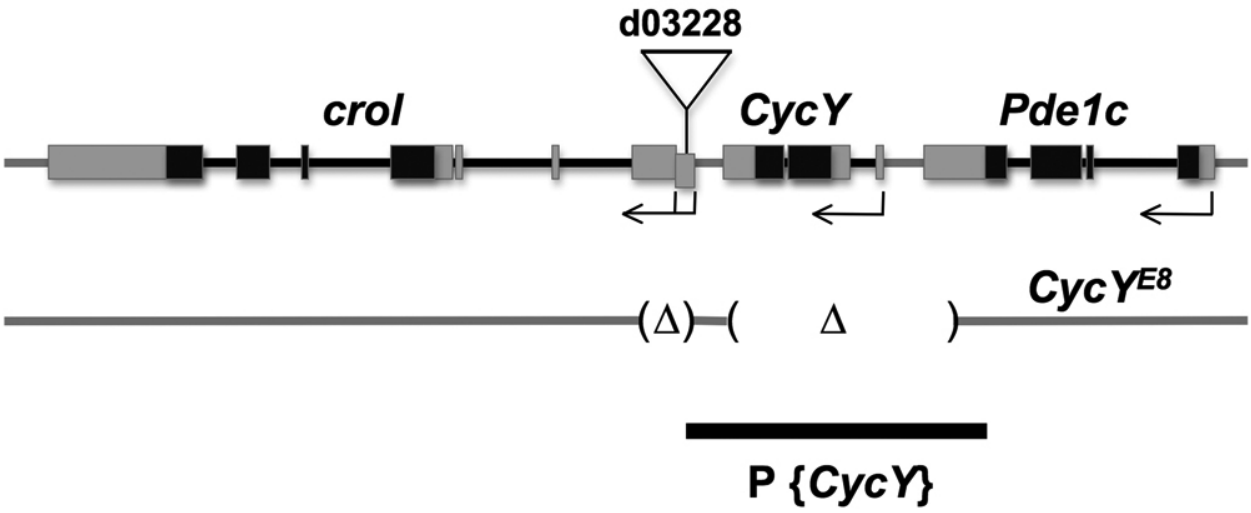


Figure 2-1. The genomic region of *CycY* and the flanking genes *crol* and *Pde1c.* Exons of the *CycY*, *crol*, and *Pde1c* transcripts are indicated by boxes. Black boxes represent coding regions and grey boxes represent untranslated regions. Direction of transcription and approximate start sites are shown with arrows. *Pde1c* has five predicted transcripts that all start from the same position. *CycY* has one predicted transcript, and *crol* has three predicted transcripts (RA, RB, RC) that start from the same position and one (RD) that starts further upstream as shown. The P-element in strain d03228 is inserted just upstream of exon 1 of *crol* transcripts RA-RC, and within exon 1 (offset box) of *crol* transcript RD. The two deleted regions in the $CycY^{E8}$ allele, which was isolated by imprecise excision of the P-element in strain d03228, are indicated as (Δ). The deletion removes the entire *CycY* gene, the first non-coding exon of *crol*, and a non-coding portion of the last exon of *Pde1c*. The genomic fragment used to create a transgene P{*CycY*} that complements $CycY^{E8}$ is also depicted (black bar).

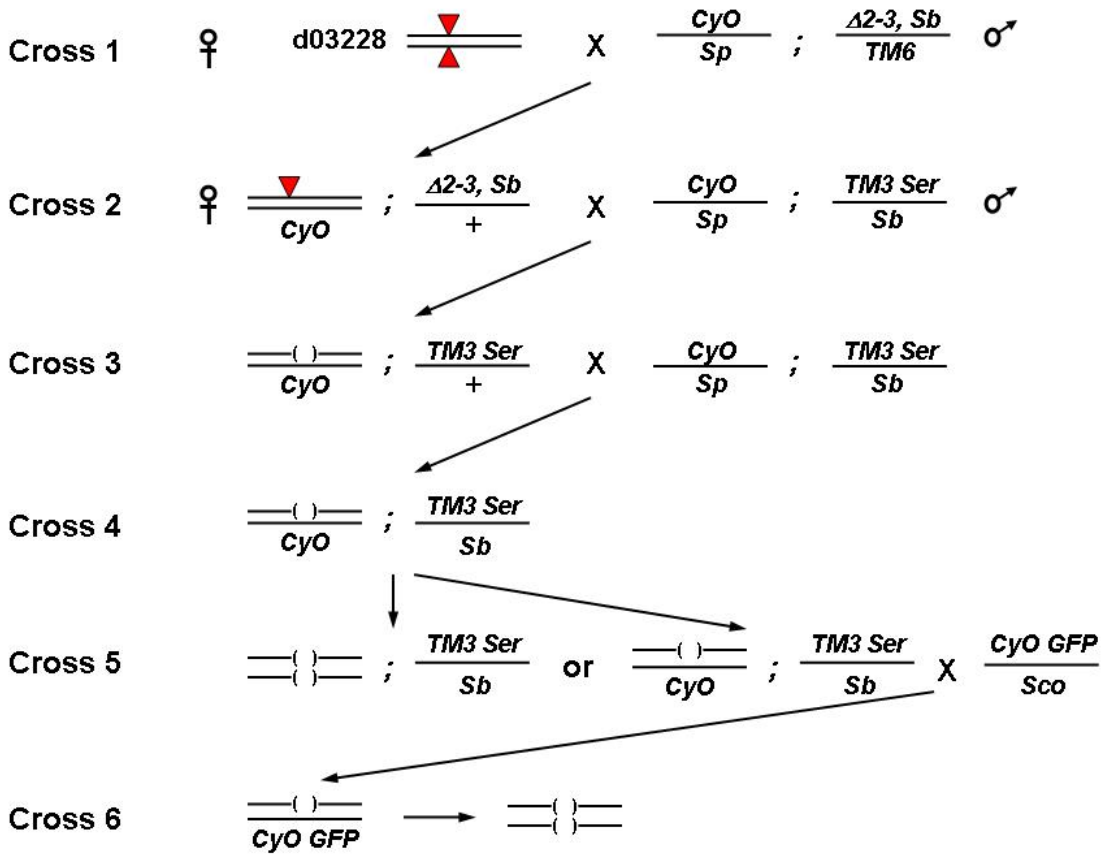


Figure 2-2. The genetic crossing scheme for performing P-element imprecise excision to generate *CycY* null alleles. Red triangles represent the P-element in strain d03228. "+" represents a wild-type chromosome. ♀, virgin females; ♂, males; ( ), a deletion; X, cross.

(Carney et al., 2004). 100 white-eyed flies were collected and further balanced individually with Finley lab # 41 to make stocks. For each stock, genomic DNA was extracted from homozygous single adult flies and analyzed by PCR for the presence of *CycY* gene regions close to the d03228 insertion site. For those stocks that are homozygous lethal, I rebalanced each line with *CyO, Act5C-GFP* balancer strain, which allows the collection of homozygous larvae (non-GFP) for genomic DNA extraction and PCR. The primer pairs used to characterize the deletion are listed in Appendix D and the positions of these primers are schematically showed in Figure 2-3. The sequences of all primers used in this study are listed in Appendix E.

The excision that removes all *CycY* coding sequences was named E8 and was shown here (Figure 2-1) to be a null allele of *CycY* ($CycY^{E8}$). To make sure there is not a second site mutation on the same chromosome as $CycY^{E8}$, the $CycY^{E8}$ chromosome was cleaned up by homologous recombination with d03228 for 7 generations. Since homozygous d03228 is fully viable and normal, I assume the P-element insertion in this line did not interfere significantly with gene expression. This makes it a good choice to be used as a wild type chromosome for homologous recombination to remove any potential second site mutations generated during mutagenesis and to avoid loss of the initial deletion while screening recombinants. The initial balanced deletion strain (*E8/CyO*) was crossed with the homozygous d03228 strain (red-eyed). I collected non-balanced orange-eyed female progeny (*E8/d03228*) and crossed again with the homozygous d03228 males. I repeated this cross for six more generations. The *E8/d03228* flies from the final cross were collected and balanced with Finley lab # 41 to make a stock. To determine the precise endpoint of the E8 deletion, genomic DNA was

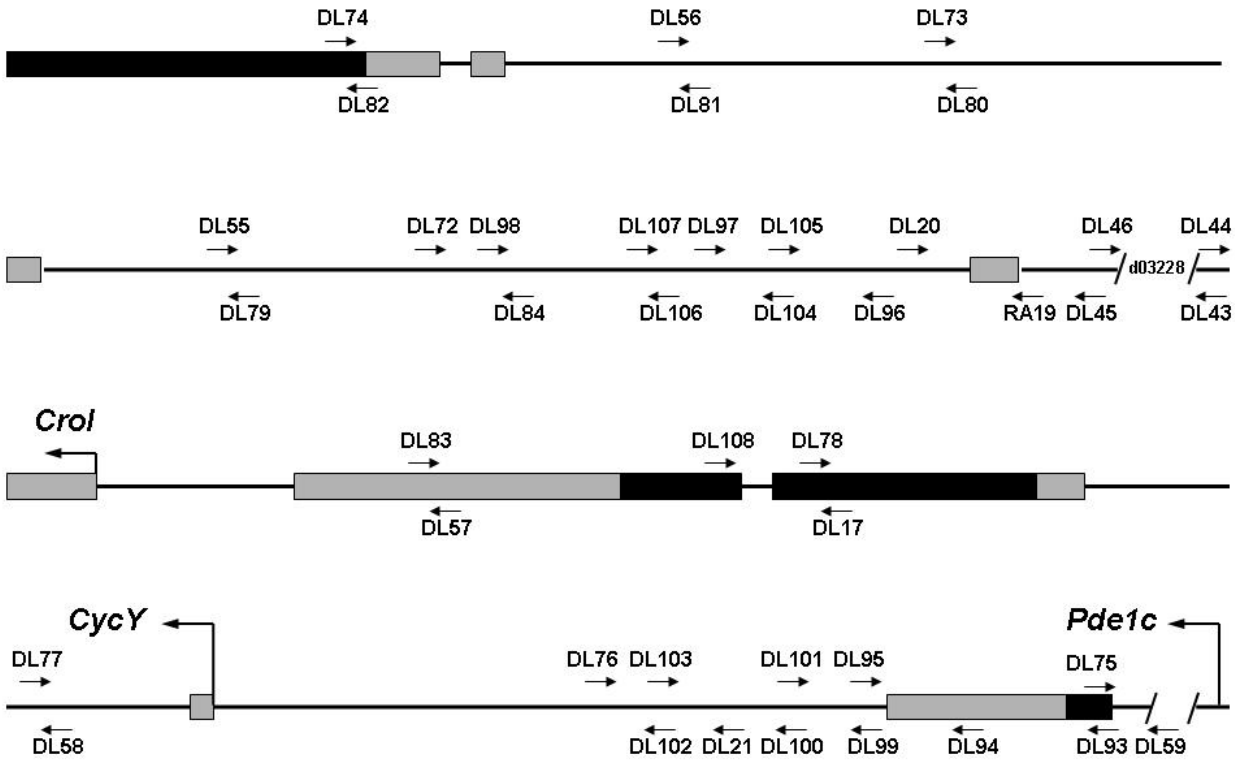


Figure 2-3. The positions of all available primers around the genomic region containing *crol*, *CycY*, and *Pde1c*. Exons of the *CycY*, *crol*, and *Pde1c* transcripts are indicated by boxes. Black boxes represent coding regions and grey boxes represent untranslated regions. Direction of transcription, approximate start sites, primer position and orientation are shown with arrows. The length of the genome is proportionate except for the region labeled with "/ /". Primer sequences are listed in Appendix E.

extracted from heterozygous *CycY*^{E8} adults and the region was amplified by PCR using primers 5'-GGGCCAAGCACAAATACAAACG-3' (DL94) and 5'-TGGTGAACGGCGAAC AGAGC-3' (DL98). The PCR product, which is about 1 kb, was gel purified and sequenced from both ends. The deletion end points were determined by sequence alignment with wild type genomic sequence. *CycY*^{E8} is missing 6,119 bp of DNA from 734 bp downstream of the *Pde1c* stop codon to 1,411 bp downstream of the *CycY* stop codon, and a second small region of 988 bp from 1,955 bp downstream of the *CycY* stop codon to the first non-coding exon of *crol* transcripts RA-RC or the first intron of *crol* transcript RD. This removes the entire *CycY* transcript along with non-coding portions of the last exon of *Pde1c* and the first non-coding exon of *crol* (Figure 2-1).

2.2.5 Extraction of genomic DNA from single flies

I followed the well established protocol by Ashburner with minor modifications (Ashburner, 1989). Freeze a single fly in an eppendorf tube at -20°C for a few minutes. Homogenize in the eppendorf tube with a pellet pestle attached to a pellet pestle cordless motor from VWR (VWR KT749521-1590 and KT749540-0000) in 50µl of homogenization buffer (10mM Tris-HCl pH7.5, 60mM NaCl, 10mM EDTA, 0.15mM Spermine, 0.15mM Spermidine, 5% Sucrose). Add 50µl of lysis buffer (300mM Tris-HCl pH 9.0, 100mM EDTA, 0.63% SDS, 5% Sucrose) and incubate the tube at 70°C heat blocker for 15 minutes. After cooling the mixture to room temperature, add 15µl of 8M KOAc and incubate on ice for 30 minutes. Centrifuge at 13500 rpm at 4°C for 5 minutes. Transfer the supernatant to a new tube and add two volumes of ethanol to precipitate DNA. After 5 minutes incubation at room temperature, centrifuge at 13500 rpm for 10

minutes at room temperature. Discard the supernatant. Air-dry the pellet for about 10 minutes and add 100μl of H_2O to dissolve the DNA. Use 1μl of the genomic DNA for PCR.

2.2.6 Lethal phase analysis

Eggs were collected from *w***; CycY*^{E8}*/CyO, Act5C-GFP* flies for 12 hours on apple juice plates with fresh yeast paste. After another 30 hours, the numbers of unhatched embryos and hatched first instar larvae were counted. Since homozygous *CyO* balancer is lethal during embryogenesis, a roughly 75% hatching rate suggests no embryonic lethality. 180 *w***; CycY*^{E8}*/CyO, Act5C-GFP* and 180 *w***; CycY*^{E8} (lacking *GFP*) first instar larvae were picked under a fluorescence dissection microscope and transferred into regular vials. The numbers of wandering third instar larvae, pupae, and adults were counted once a day for 15 days to score for a delay in puparium formation, progression through metamorphosis, and adult eclosion. To estimate the delay of puparium formation more accurately, I followed 180 first instar larvae for each genotype and calculated the average time to form pupa by counting the number of pupa newly formed after each 24 hour period and averaging over all individuals and days. Similarly, I analyzed the following animals: (1) *w***; CycY*^{E8}*/CyO, Act5C-GFP; P{CycY},* (2) *w***; CycY*^{E8}*; P{CycY},* (3) *w***; CycY*^{E8}*/CyO, Act5C-GFP* and *Df(2L)Exel6030*/*CyO, Act5C-GFP*, (4) *w***; CycY*^{E8}/*Df(2L)Exel6030*, (5) *w***; CycY*^{E8}*/CyO, Act5C-GFP*; *P{CycY}* and *w***; Df(2L)Exel6030*/*CyO, Act5C-GFP*; *P{CycY}*, (6) *w***; CycY*^{E8}/*Df(2L)Exel6030*; *P{CycY}*, (7) *w***; Eip63E*^{81}*/TM3, Ser Act5C-GFP* and *w***; Eip63E*^{GN50}*/TM3, Ser Act5C-GFP,* (8) *w***; Eip63E*^{81}*/Eip63E*^{GN50}. All flies were incubated at 25ºC except were noted.

2.2.7 Phenotypic characterization

200 *w**; *CycY*^{E8}*/CyO, Act5C-GFP* and 200 *w**; *CycY*^{E8} first instar larvae were collected (see above), transferred into individual fresh vials and allowed to develop for 9 days. For each genotype, pupae at all developmental stages were collected, weighed, and imaged. The relative pupal length was measured based on the image size. The average pupa weight and length were then calculated. To document the pupal phenotype, pupae at all developmental stages were carefully removed from the wall. For pharate adults, the pupal case was gently dissected. Images were taken with the Leica MZ 16FA Stereomicroscope and Leica DFC 490 camera (kindly provided by Dr. Markus Friedrich). Similarly, I analyzed the following animals: (1) *w**; *CycY*^{E8}*/CyO, Act5C-GFP; P{CycY},* (2) *w**; *CycY*^{E8}*; P{CycY},* (3) *w**; *CycY*^{E8}*/CyO, Act5C-GFP* and *Df(2L)Exel6030*/*CyO, Act5C-GFP*, (4) *w**; *CycY*^{E8}/*Df(2L)Exel6030*, (5) *w**; *CycY*^{E8}*/CyO, Act5C-GFP*; *P{CycY}* and *w**; *Df(2L)Exel6030*/*CyO, Act5C-GFP*; *P{CycY}*, (6) *w**; *CycY*^{E8}/*Df(2L)Exel6030*; *P{CycY}*, (7) *w**; *Eip63E*^{81}*/TM3, Ser Act5C-GFP* and *w**; *Eip63E*^{GN50}*/TM3, Ser Act5C-GFP,* (8) *w**; *Eip63E*^{81}/*Eip63E*^{GN50}.

2.2.8 Heat shock induction and rescue efficiency

Eggs from a self cross of *CycY*^{E8}*/CyO; hs-CycY/TM3, Ser* flies (Finley lab fly stock # 700) were collected in glass vials with standard cornmeal Drosophila medium for 24 hours and then heat shocked on each day as indicated in Figure 2-12. For each heat shock induction, glass vials were incubated in a 37ºC water bath for one hour. Vials were kept at 25ºC otherwise. The numbers of flies with or without curly wings were

counted separately. If *CycY^{E8}/CyO* and *CycY^{E8}* flies have equal viability (full rescue), the number of *CycY^{E8}* flies should be half of that of *CycY^{E8}/CyO* flies. The rescue efficiency was then determined by the number of *CycY^{E8}* adult flies divided by half of the number of *CycY^{E8}/CyO* adult flies. The genotype of representative flies was confirmed by single-fly PCR. The primers used to confirm the genotypes were listed in Table 2-1.

2.2.9 Generation of mosaic germline clones with homozygous *CycY^{E8}*

CycY^{E8} was recombined with *FRT40A* as previously described (Xu and Rubin, 1993). The genetic crossing scheme is shown in Figure 2-4. *CycY^{E8}* males or virgin females (Finley lab stock # 692) were crossed with *FRT40A* flies with opposite sex (Finley lab stock # 355). Virgin females with straight wings and red eyes from the above cross were then mated with balancer strain males (Finley lab stock # 41). Individual red-eyed males with curly wings from the above cross were mated with *CycY^{E8}* virgin females (Finley lab stock # 692). If none of the progeny had straight wings from the above cross, it suggested that *CycY^{E8}* successfully recombined with *FRT40A* since homozygous *CycY^{E8}* is lethal (see Results and Discussion). I collected red-eyed adults with curly wings to make a stock (Finley lab stock # 702).

Germline clones with homozygous *CycY^{E8}* were generated based on the well-established approach of Chou et al. with minor modification (Chou and Perrimon, 1996). *w*; CycY^{E8} FRT40A/CyO* females were crossed with *hs-FLP/Y; ovo^{D1} FRT40A/CyO* males for 3 days. Eggs were collected for 3 days and aged for 2 more days. Larvae, which were at either second or third instar stages, were heat shocked at 37ºC in a water bath for two hours. Females with straight wings (*hs-FLP/w*; CycY^{E8} FRT40A/ovo^{D1}*

Table 2-1. Primers used to confirm the genotypes.

Genotypes	Primer pairs	Product length
CycY^{E8}/CyO; hs-CycY/TM3, Ser	DL94/DL98	1 kb
	DL93/DL101	1.15 kb
CycY^{E8}; hs-CycY/TM3, Ser	DL94/DL98	1 kb
	DL93/DL101	negative

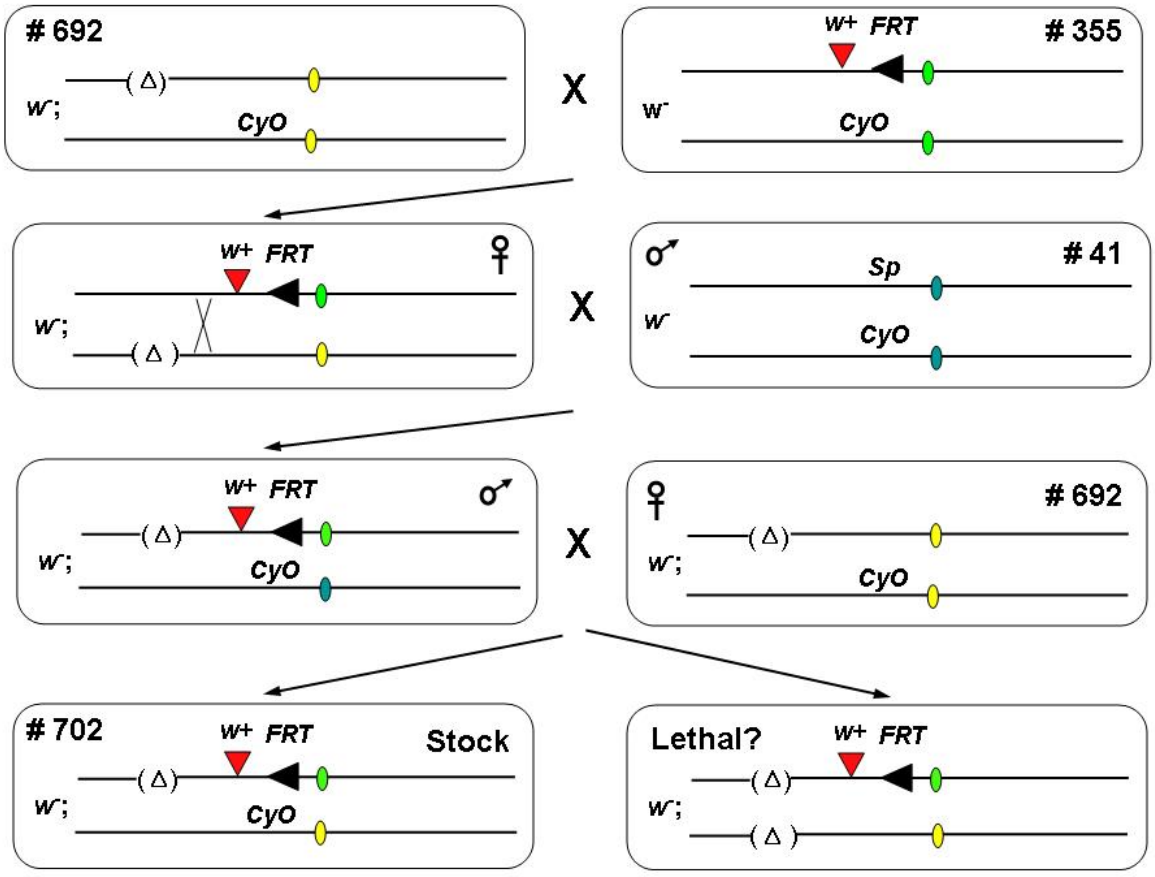


Figure 2-4. The crossing scheme for recombining *CycY^{E8}* with *FRT40A*. Red triangles represent a P-element with the *miniwhite* gene (38D4) closely linked to the *FRT40A* insertion used as a selection marker for *FRT40A*; Black triangles represent the *FRT40A* site; ♀, virgin females; ♂, males; (Δ), a deletion; X, cross. Bigger X, recombination; Oval, centromere.

FRT40A) from the above cross were then mated to *CycY*^{E8}*/CyO, Act5C-GFP* males to test for a maternal requirement for *CycY*. *GFP* positive (*CycY*^{E8} *FRT40A/CyO, Act5C-GFP)* and *GFP* negative (*CycY*^{E8} *FRT40A/CycY*^{E8}) first instar larvae were picked and development was followed as described above.

2.2.10 Gene expression

Gene expression was assayed by reverse-transcription PCR (RT-PCR) (Figure 2-7) or quantitative real-time PCR (RT-qPCR) (Figure 2-13). Flies at the indicated developmental stages were collected and total RNA was extracted using the RiboPure® Kit (Ambion). The RNA samples were then treated with DNase from a DNA-free Kit (Ambion) to remove contaminating genomic DNA. cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. qPCR reactions were performed using Brilliant® SYBR® Green QPCR Master Mix (Stratagene) in a 96-well plate. qPCR reactions were carried out in triplicate for each RNA sample. The primers used in this work are listed in Table 2-2. *rp49* was used as the internal control gene and the mRNA level of *CycY* was normalized to *rp49* levels.

2.2.11 Co-affinity purification (co-AP) assays

Co-AP assays were conducted by expressing pairs of N-terminally myc-tagged and NTAP-tagged proteins in Drosophila S2R+ cells, purification of the NTAP-tagged protein, and detection of associated myc-tagged proteins by immunoblotting. Myc-tagged proteins were expressed from pAS1. NTAP-tagged proteins were expressed from pDL4, a derivative of pUAST-NTAP (Veraksa et al., 2005) containing the NTAP tag

Table 2-2. Primers used for RT-PCR and qPCR.

Genes	Primers Finley lab # (alias)	Sequence	Position[a]	Product length (bp)
CycY	722 (DL108)	5'-AGGAGAATGGCACCCAAC	765-782	414
	469 (DL17)	5'-TACTCCCGGTGGCAATAG	1161-1178	
crol	723 (DL109)	5'-AGCTCGGTGCCATCAGTAG	1440-1458	332
	724 (DL110)	5'-GCGGCATTATTCGTGGACG	1753-1771	
Pde1c	728 (DL114)	5'-GTGTGATCGCAACAATACGC	1622-1641	465
	729 (DL115)	5'-TTGCTTTCCTCCGCTTCC	2069-2086	
β-Tubulin	642 (DL12)	5'-GACCATGTCCGGCGTAAC	881-898	438
	643 (DL13)	5'-AGCTCCTGGATGGCAGTG	1301-1318	
rp49	732 (DL118)	5'-GATATGCTAAGCTGTCGCACAAATGGC	95-121	118
	733 (DL119)	5'-GTGCGCTTGTTCGATCCGTAACCG	189-212	

[a] Inclusive nucleotide positions in predicted transcript RA for each gene.

followed by the same 5' and 3' RTs found in pAS1. Note that pDL4 was validated for Gal4-dependent expression of NTAP-tagged proteins in transiently-transfected Drosophila S2R+ cells, but it cannot be used to make transgenic flies, possibly due to mutations in the P-element. Coding regions from the ATG to the stop codon of various Cdks or cyclins were amplified from yeast two-hybrid clones (Stanyon et al., 2004) with primers that added the 5' and 3' RTs and the products were subcloned into pAS1 or pDL4 by gap repair in *E.coli*. I used the following protocols adapted from Effectene user's manual and Veraksa's protein purification protocol for transfection, cell lysis, and co-AP assays (Veraksa et al., 2005).

2.2.11.1 Transfection of cultured Drosophila cells

Day 1 afternoon: Resuspend S2R+ cells in a T75 flask by pipetting and count the cell numbers. Dilute the cell suspension to $1x10^6$ cells/ml with fresh Schneider's Drosophila culture media (Invitrogen) supplemented with 10% FBS and 0.1mg/ml Gentamicin. Seed 2ml of the above diluted cells in each well of a 6-well plate. Incubate in the 25°C fly incubator overnight. Day 2 afternoon about the same time: Replace the old media in the 6-well plate with fresh media and put it back into the incubator until transfection. For each transfection, mix 10µl of 50ng/µl pAS1-X, 10µl of 50ng/µl pDL4-Y, and 10µl of 50ng/µl pMT-Gal4 (Klueg et al., 2002). Add 70 µl of buffer EC to make a total of 100 µl. Add 12µl of enhancer, vortex at the highest speed for 1 second, and incubate at room temperature for 5 minutes. Add 30 µl of effectene, vortex at the highest speed for 15 seconds, and incubate at room temperature for 10 minutes. Mix with 600µl of culture media and add to the top of the cells in a drop-wise way. Put the plate back into the fly incubator. The reagents mentioned above, including buffer EC,

enhancer, and effectene, are from the effectene transfection kit (Qiagen). Day 3 morning: Add 220µl of 10mM $CuSO_4$ to each well to induce the expression of Gal4. Day 4 morning: Replace with 1.5ml of fresh media supplemented with 1mM $CuSO_4$ in each well. Day 6: Resuspend the transfected cells by pipetting up and down several times. Transfer the cells to a 1.5ml of eppendorf tube and centrifuge at 13500 rpm for 2 minutes at 4°C to spin down the cells. Wash the cell pellet two times with cold PBS.

2.2.11.2 Cell lysis

Add 300 µl of fresh-made lysis buffer [50mM Tris-HCl pH7.4, 180mM NaCl, 5mM EDTA, 1% NP-40, 10% glycerol, 50mM NaF, 1mM Na_3VO_4, 1mM PMSF, 1x Protease inhibitor cocktail (Roche)] to the cell pellet from one well of the 6-well plate. Homogenize by pipetting up and down several times. Incubate on ice for 45 minutes. Vortex once every 5 minutes. Centrifuge at 13500 rpm at 4°C for 40 minutes to clarify the cell lysate. Transfer the supernatant to a new tube.

2.2.11.3 Protein quantification

Quantify the protein concentration using the BioRad protein assay reagent. Generate a standard curve using a series of BSA dilutions ranging from 0 to 10µg/ml. Take 4µl of protein lysate and add 796µl of H_2O to make a total of 800µl. Mix with 200µl of BioRad protein assay dye reagent. Incubate at room temperature for 5 minutes and then measure OD_{595} to determine the absorbance and convert it to the protein concentration on the basis of the standard curve. Usually, the protein concentration is about 1.5-2.5µg/µl.

2.2.11.4 Western blot to examine the protein expression

Mix the cell lysate with LDS sample loading buffer (Invitrogen) and incubate at 70°C for 10 minutes. Load 15μg of total protein for myc western blot or 2μg for protein A western blot. Run the SDS-PAGE gel with Invitrogen electrophoresis system at 200V for 30-40 minutes. Transfer the protein to PVDF membrane (Bio-Rad) at 40V for 90 minutes. Block with 1XPBS, 5% milk at room temperature for one hour or 4°C overnight. Incubate with primary antibody (anti-myc 1:500, Santa Cruz) at room temperature for one hour or 4°C overnight. Wash with 1XPBS 2 times. Incubate with secondary antibody [goat anti-mouse HRP conjugated secondary antibody (1:10000, BioRad) or goat anti-Protein A peroxidase-conjugated antibody (1:15000, Rockland Immunochemicals)] at room temperature for one hour. Wash with 1XPBS, 0.05% Tween-20 5 times for 10 minutes each time. Detect proteins with ECL plus reagents (GE Healthcare Life Sciences).

2.2.11.5 Co-AP

Transfer 40μl of rabbit IgG-conjugated agarose beads (Sigma) slurry (20μl of settled beads) into an eppendorf tube using big orifice pipet tips. Wash 3 times with lysis buffer. Mix with 500μg of cell lysate in a total of 1ml of lysis buffer. Incubate at 4°C for two hours. Wash with lysis buffer 5 times. Add 1XLDS sample buffer to the beads and heat at 70°C for 10 minutes. Resolve co-purified proteins by SDS-PAGE and detect by western blotting.

2.2.12 Sequence alignments

We determined the reciprocal best-match BLAST hits between Drosophila and human cyclins (Table 2-3). BLAST searches were conducted with each of the Drosophila cyclins listed below to identify the top matching human cyclins. In cases where a gene had multiple protein isoforms, the longest isoform that had a cyclin domain was used. The top matching human proteins were then used in BLAST searches against the *Drosophila melanogaster* annotated proteins and the top matching protein was identified. Reciprocal best-match BLAST hits are listed in Table 2-3. An example of how reciprocal best-match hits are interpreted is as follows: Human CCND1, CCND2, and CCND3 are the human proteins most similar to Drosophila CycD, and Drosophila CycD is the Drosophila protein most similar to human CCND1, CCND2, or CCND3, according to BLAST. Multiple sequence alignment was performed using ClustalX version 2 (Larkin et al., 2007; Thompson et al., 2002). Pair-wise percent identity was determined by dividing the number of identical sites in the alignment by the length of the alignment, including gaps and unaligned ends. The dendrogram shown in Figure 2-5 A was constructed using ClustalX with the neighbor-joining algorithm. For Figure 2-5 B, the reciprocal best-match BLAST hits between Drosophila CycY and proteins from several divergent species were aligned using ClustalW followed by manual corrections to improve identities. Only the top matching CycY-like protein from each species is shown. The proteins aligned were as follows, where Genbank accession numbers are in parentheses: *Aedes aegypti* hypothetical protein AaeL_AAEL010543 (XP_001660900.1); *Caenorhabditis elegans* hypothetical protein ZK353.1a (NP_498858.2); *Danio rerio* hypothetical protein LOC767752 (NP_001070188.1); *Drosophila melanogaster* CG14939-PA (NP_609519.1); *Gallus*

Table 2-3. Drosophila-Human reciprocal best-match proteins.

Drosophila protein	Genbank ID	Human Gene	Human protein	Genbank ID
CG14939-PA (CycY)	NP_609519	*CCNY*	Cyclin Y	NP_659449.3
CG14939-PA (CycY)	NP_609519	*CCNYL1*	Cyclin Y-like 1	NP_689736.1
CycA-PA	NP_524030	*CCNA1*	Cyclin A1	NP_001104516
CycA-PA	NP_524030	*CCNA2*	Cyclin A2	NP_001228.1
CycB-PB	NP_726246	*CCNB1*	Cyclin B1	NP_114172.1
CycB-PB	NP_726246	*CCNB1*	Cyclin B2	NP_004692.1
CycB3-PA	NP_651303	*CCNB3*	Cyclin B3	NP_149020.2
CycC-PA	NP_476848	*CCNC*	Cyclin C	NP_005181.2
CycD-PF (PC)	NP_727913.1	*CCND2*	Cyclin D2	NP_001750.1
CycD-PF (PC)	NP_727913.1	*CCND3*	Cyclin D3	NP_001751.1
CycD-PF (PC)	NP_727913.1	*CCND1*	Cyclin D1	NP_444284.1
CycE-PD	NP_723925	*CCNE1*	Cyclin E1	NP_001229.1
CycE-PD	NP_723925	*CCNE2*	Cyclin E2	NP_477097.1
CycG-PC	AAF57169.2	*CCNG1*	Cyclin G1	NP_004051.1
CycG-PC	AAF57169.2	*CCNG2*	Cyclin G2	NP_004345.1
CycH-PA	NP_524207	*CCNH*	Cyclin H	NP_001230.1
CycJ-PA	NP_523903	*CCNJ*	Cyclin J	NP_001127847.1
CG16903-PA	NP_569980	*CCNL2*	Cyclin L2	NP_112199.2
CG16903-PB	NP_569980	*CCNL1*	Cyclin L1	NP_064703.1
CycK-PB	NP_788083	*CCNK*	Cyclin K	NP_001092872.1
CycT-PB	NP_524127	*CCNT2*	Cyclin T2	NP_001232.1
CycT-PB	NP_524127	*CCNT1*	Cyclin T1	NP_001231.2
Koko-PA	NP_650721	*FAM58A*	Family 58A	NP_689487.2
Koko-PA	NP_650721	*FAM58B*	Family 58B	NP_001098987.1

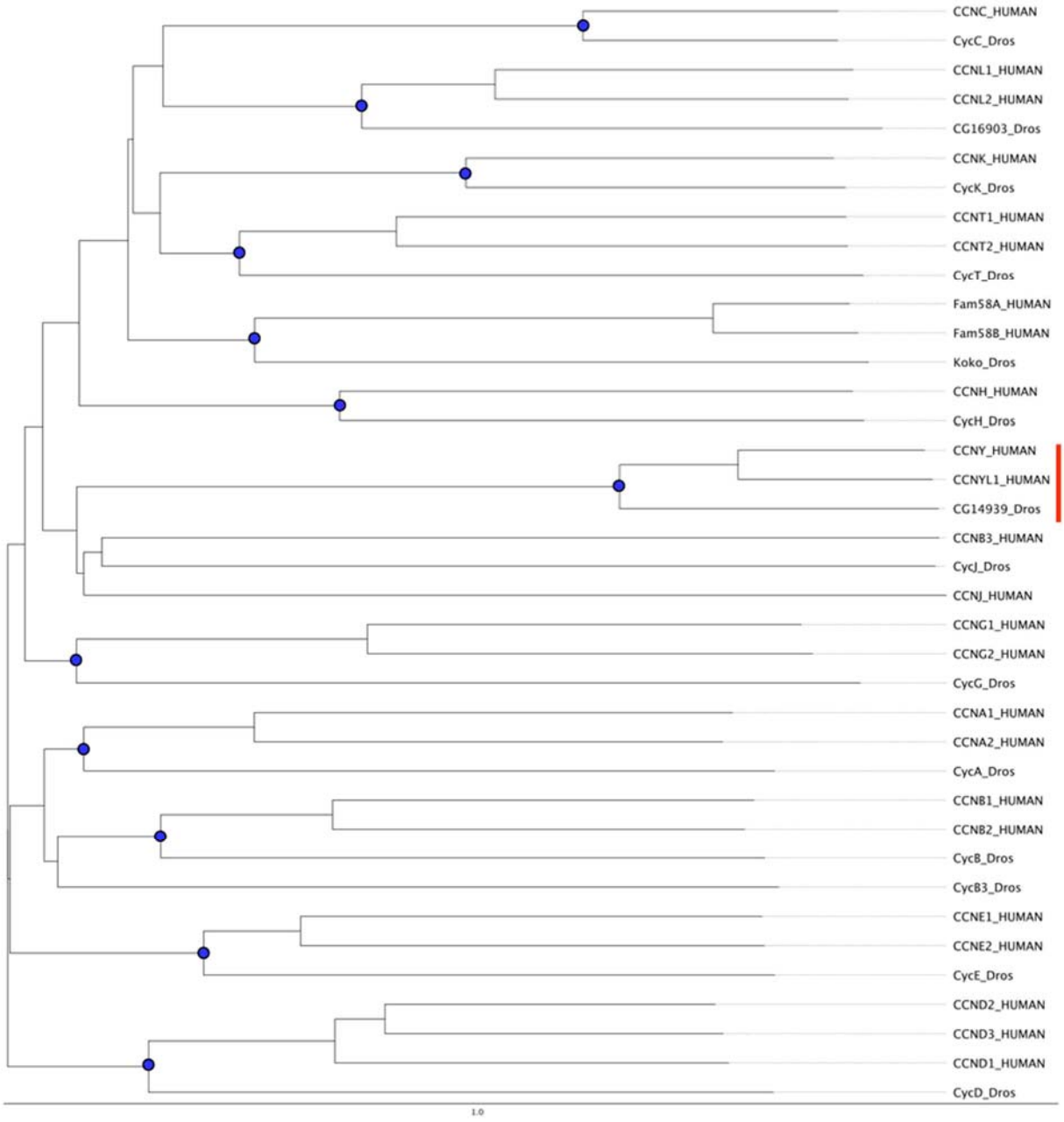


Figure 2-5. ***CG14939* encodes a highly conserved Y-type cyclin. (A)** Phylogenetic tree resulting from alignment of all Drosophila cyclins and cyclin-like proteins and their corresponding human orthologs. Blue dots at branch points indicate that the attached nodes (proteins) are reciprocal best-match BLAST hits between Drosophila and human (see Materials and Methods). Lengths of the horizontal lines between nodes and branch points indicate relative sequence similarity; e.g., the human and Drosophila Y-type cyclins (red line) are more similar to each other than are any other human and Drosophila cyclins except for CycC.

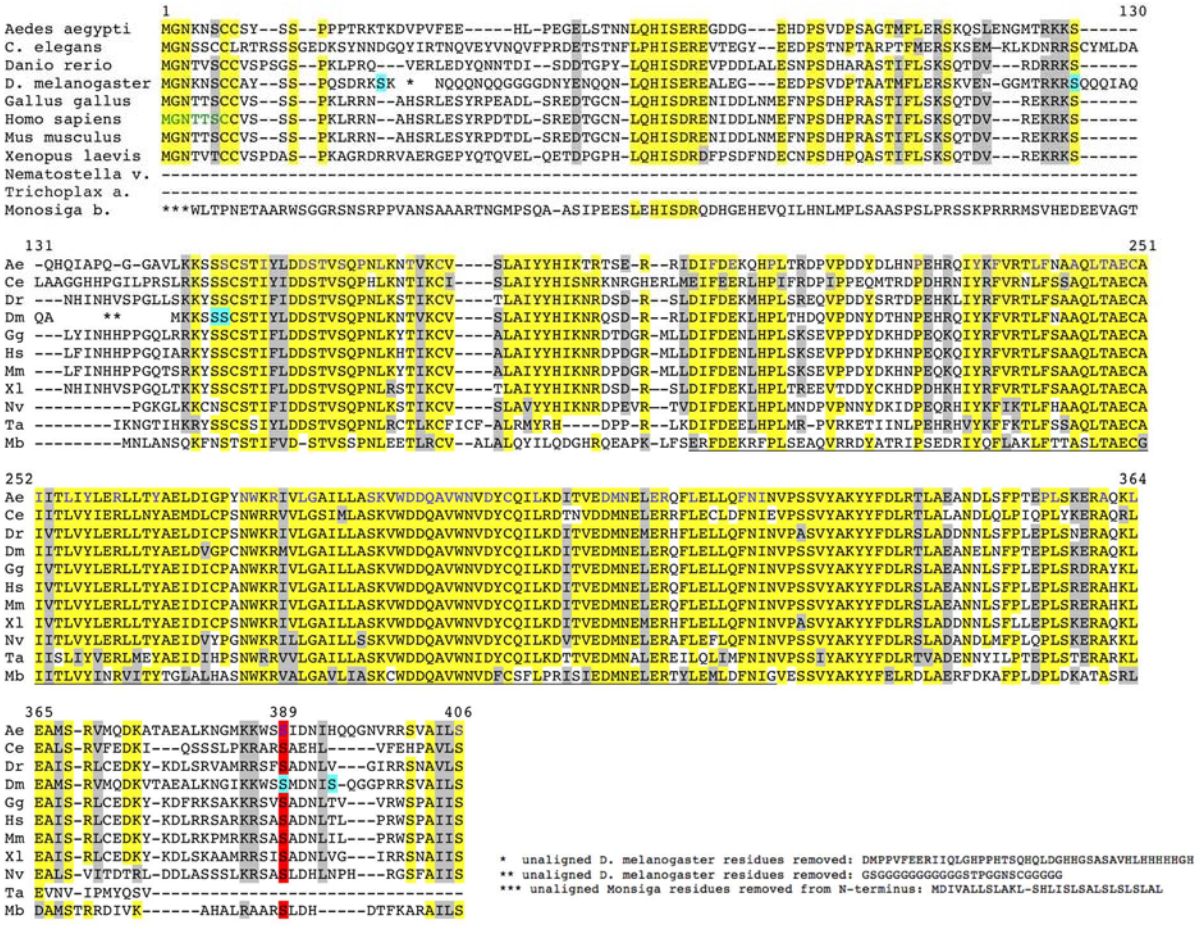


Figure 2-5. ***CG14939* encodes a highly conserved Y-type cyclin. (B)** Alignment of Drosophila CycY and the most similar proteins from several other species. The sequences available for *Nematostella* and *Trichoplax* may be truncated because the genome sequences were still in draft form. Yellow or grey shaded amino acids are identical or similar, respectively, in at least 8 out of the 11 species shown, or 7 of 9 species where the N- and C-terminal sequences of *Nematostella* and *Trichoplax* appear to be missing. Blue-shaded amino acids in the *D. melanogaster* sequence are known to be phosphorylated in embryos (Zhai et al., 2008). Red-shaded serines, corresponding to S389 in *D. melanogaster*, are highly conserved and phosphorylated in both Drosophila and human CycY (Beausoleil et al., 2004; Olsen et al., 2006; Zhai et al., 2008). The N-terminal region of *H. sapiens* CycY contains a putative myristoylation signal (green lettering), previously noted by Jiang et al., (Jiang et al., 2009), which appears to be conserved in many other species. All of the sequences contain the conserved cyclin domain (underlined), corresponding to amino acids 205 to 328 of Drosophila CycY; this domain is annotated in these sequences by the Conserved Domain Database (Marchler-Bauer et al., 2009) and corresponds to pfam (Finn et al., 2009), domain pfam:00134, “Cyclin_N”, the N-terminal cyclin fold found in the cyclin superfamily. Dashes indicate gaps in the alignment. Asterisks in the *D. melanogaster* sequence indicate unaligned residues that were removed and are shown below the alignment; one sequence is histidine-rich and the other is glycine-rich, and neither appears to be conserved. The unaligned N-terminal region of the *Monsiga brevicollis* sequence is also shown below. Numbers above the lines indicate residue numbers for the Drosophila protein. Gene names are listed in Materials and Methods.

gallus CCNYL1 cyclin Y-like 1 (XP_425973.2); *Homo sapiens* cyclin fold protein 1 variant b (AAL78999.1); *Mus musculus* cyclin fold protein 1 (NP_080760.2); *Xenopus laevis* hypothetical protein LOC431857 (NP_001084816.1); *Nematostella vectensis* predicted protein (XP_001641126); *Trichoplax adhaerens* hypothetical protein (XP_002116466); *Monosiga brevicollis* hypothetical protein (XP_001750168).

To identify proteins with similarity to CycY in more distant species, reciprocal best-match BLAST hits between Drosophila CycY and proteins in the species listed below were determined. The identified proteins were also determined to be reciprocal best-match BLAST hits with the human *CCNY* protein. For all of the identified proteins the sequence similarity with the human or Drosophila *CycY* proteins was restricted to the annotated cyclin domain (Marchler-Bauer et al., 2009) and immediate flanking regions, referred to as the “cyclin+” region in Figure 2-6. The cyclin+ regions were aligned using ClustalW and a consensus sequence was determined by identifying residues that were found in >50% of the sequences (Figure 2-6A). The dendrogram shown in Figure 2-6B was obtained by aligning the cyclin+ region of the proteins most similar to CycY, and the annotated cyclin domains of reciprocal best-match hits of Drosophila CycA and CycB for the species shown. Only the top matching CycY-like protein from each species is shown; gene or genome duplications in some lineages have resulted in several parologous CycY-like proteins (not shown). The following proteins from non-metazoan species were reciprocal best-match hits of Drosophila CycY or the human *CCNY* protein, where Genbank accession numbers are in parentheses: *Arabidopsis thaliana* CYCP4;3 (NP_196362.1); *Coprinopsis cinerea* predicted protein (XP_001832875); *Cryptococcus neoformans* cyclin (XP_566770);

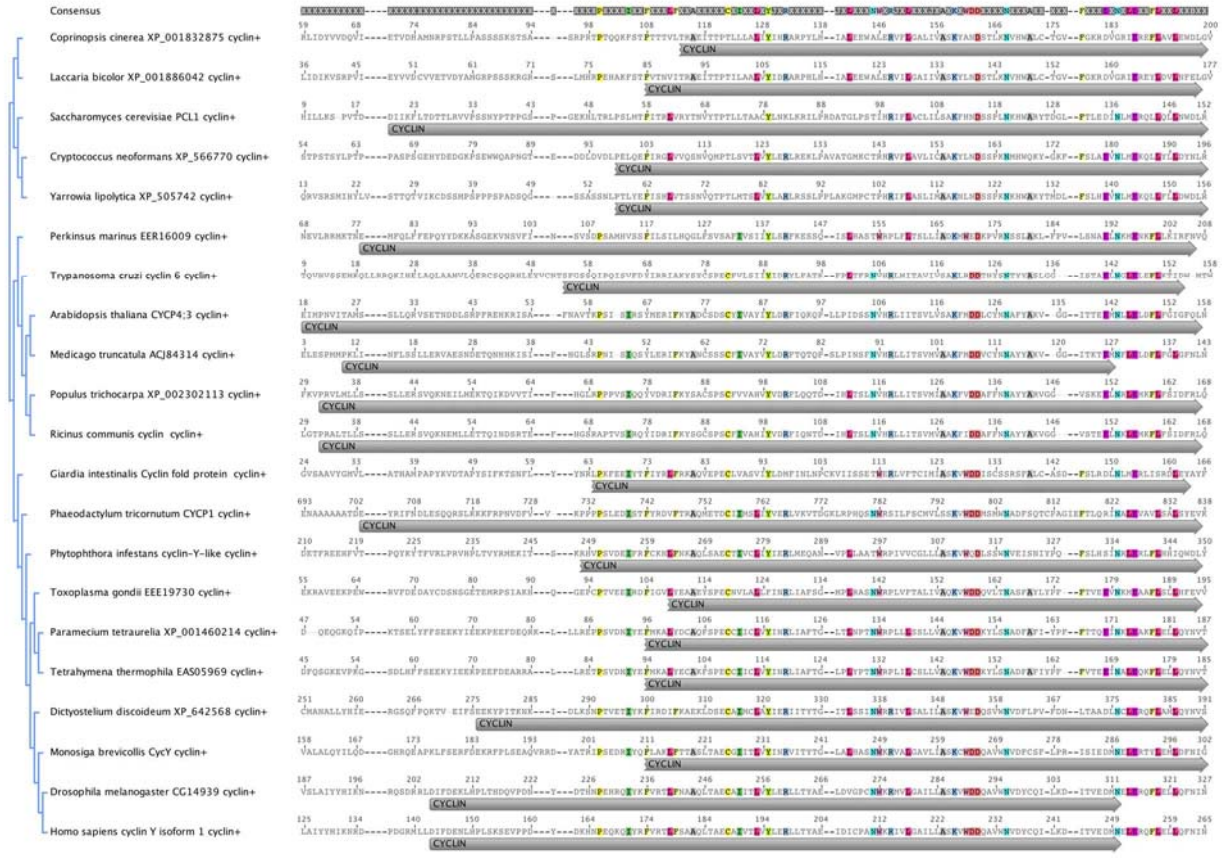


Figure 2-6. **The cyclin domain of Y-type cyclins is novel and conserved throughout the eukaryotic kingdom.** (**A**) Alignment of the cyclin domains from the proteins that are reciprocal best-match BLAST hits of Drosophila CycY in many non-metazoan species. These proteins are also reciprocal best-match BLAST hits of human CycY. Alignments include the cyclin domains (arrows) as annotated by the Conserved Domain Database (Marchler-Bauer et al., 2009) along with the indicated flanking region of each protein. A consensus sequence was obtained as 31 residues that are identical in at least 50% of the proteins (colored); the Drosophila and human proteins each share 27 of these consensus residues. Only the top related protein from each species is shown.

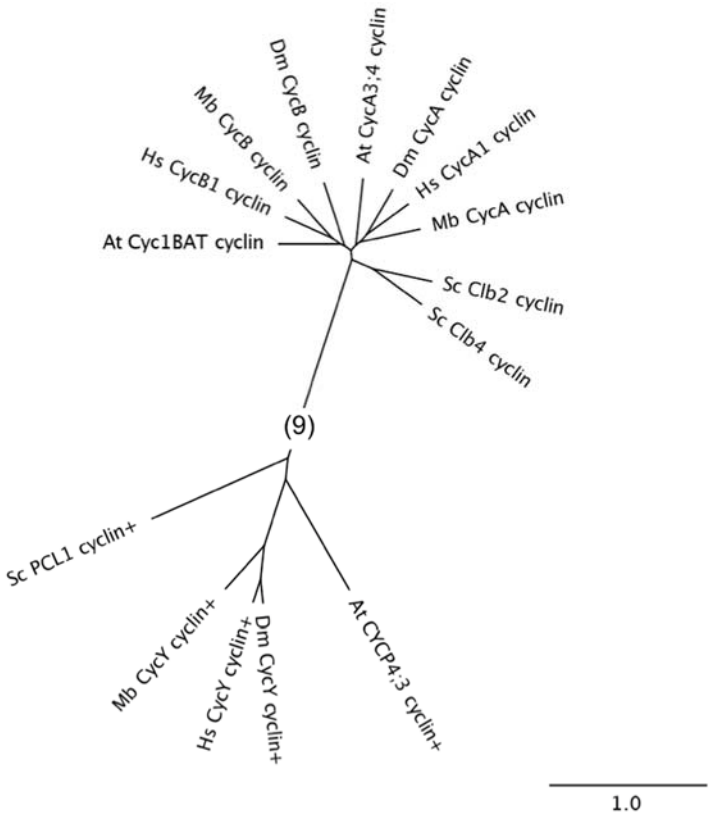


Figure 2-6. **The cyclin domain of Y-type cyclins is novel and conserved throughout the eukaryotic kingdom.** (**B**) Dendrogram showing sequence similarity among the cyclin domains from several distant species. Cyclin domains from *Drosophila melanogaster* (Dm) CycA, CycB, and CycY and their reciprocal best-match BLAST hits in human (Hs), *Monosiga brevicollis* (Mb), *Arabidopsis thaliana* (At), and *Saccharomyces cerevisiae* (Sc) were aligned. The cyclin domains from the Y-type cyclins included the annotated cyclin domain and small flanking regions as shown in Figure 2-6A (cyclin+). Only the top related protein from each species is shown. The length of the lines is proportional to sequence similarity. The lower cluster of Y-type cyclin domains and the upper cluster of A and B-type cyclin domains are separated by a relative distance of 9 (see scale bar for relative distances).

Dictyostelium discoideum cyclin domain-containing protein (XP_642568); *Giardia intestinalis* Cyclin fold protein 1 (EET00183.1); *Laccaria bicolor* predicted protein (XP_001886042); *Medicago truncatula* unknown (ACJ84314); *Paramecium tetraurelia* hypothetical protein (XP_001460214); *Perkinsus marinus* hypothetical protein (EER16009); *Phaeodactylum tricornutum* CYCP1 (XP_002182703.1); *Phytophthora infestans* cyclin-Y-like (EEY67633.1); *Populus trichocarpa* predicted protein (XP_002302113); *Ricinus communis* cyclin (XP_002520742.1); *Saccharomyces cerevisiae* PCL1 (NP_014110.1); *Tetrahymena thermophila* Cyclin, N-terminal domain containing protein (EAS05969); *Toxoplasma gondii* cyclin, N-terminal domain-containing protein (EEE19730); *Trypanosoma cruzi* cyclin 6 (AAG44389.1); *Yarrowia lipolytica* hypothetical protein (XP_505742).

2.2.13 Yeast two-hybrid assays

Yeast two-hybrid assays (Fields and Song, 1989) were performed using the LexA system (Gyuris et al., 1993) and interaction mating assays (Finley and Brent, 1994). Yeast strains and vectors, the protocol for one-on-one mating assays, and the reporter scoring methods were previously described (Zhong et al., 2003). All of the cyclins tested were expressed as activation domain (AD) fusions, whereas all of the Cdks were expressed as DNA-binding domain (BD) fusions. AD and BD strains were obtained from the arrays of LexA-based yeast two-hybrid clones previously described (Stanyon et al., 2004).

2.2.14 Tandem affinity purification (TAP)

Tandem affinity purification was performed as previously described (Puig et al., 2001; Veraksa et al., 2005). Drosophila S2R+ cells were cotransfected with pDL4-CycY and pMT-Gal4 in a 100mm dish. Cells were induced with $CuSO_4$, harvested, and lysed as described above. About 10-15mg clarified cell lysate was incubated with 100μl of settled IgG agarose beads (Sigma A2909) at cold room for 2 hours on a nutator. The lysate/beads mixture was loaded to a poly-prep column (0.8 x 4 cm, BioRad 731-1550), which was then washed 3 times with 10ml of lysis buffer and once with 10ml of TEV cleavage buffer (10mM Tris.HCl pH8.0, 150mM NaCl, 0.1% NP-40, 0.5mM EDTA, 1mM DTT). 100 units of AcTEV protease (Invitrogen 12575-023) in 1ml of TEV cleavage buffer was added to the beads and the mixture was incubated at 16°C for two hours. Three volumes of calmodulin binding buffer (10mM β-mercaptoethanol, 10mM Tris.HCl pH8.0, 150mM NaCl, 1mM Mg-acetate, 1mM imidazole, 2mM $CaCl_2$, 0.1% NP40) and 3/1000 volume of 1M $CaCl_2$ were added to the elute from TEV cleavage to promote the binding with calmodulin beads and titrate the EDTA coming from the TEV cleavage buffer. 100μl of settled calmodulin beads were incubated with the above elute at 4°C for 2 hours. Beads were then washed with 4ml of calmodulin binding buffer and finally, purified proteins were eluted from the beads with 400μl of calmodulin elution buffer (10mM β-mercaptoethanol, 10mM Tris.HCl pH8.0, 150mM NaCl, 1mM Mg-acetate, 1mM imidazole, 2mM EGTA, 0.1% NP40). Concentrated elute was sent to the University of Michigan, Michigan Proteome Consortium, for MALDI/MS-MS.

2.3 RESULTS AND DISCUSSION

2.3.1 CycY is a conserved uncharacterized cyclin

Drosophila *CG14939* has a single predicted transcript that encodes a protein with 406 residues (Figure 2-1, 2-5B). Between amino acids 205 and 328 lies a cyclin domain, a conserved region that defines the cyclin family of proteins. The closest human homolog of *CG14939* is a poorly characterized gene called *Cyclin Y* (*CCNY*). Genes in a number of other species have also been named *Cyclin Y* based on their sequence similarity to human *CCNY*. *CG14939* is more similar to the Y cyclins from other species than it is to any other *Drosophila melanogaster* gene (Figure 2-5) indicating that it belongs to this orthologous family of proteins. We therefore renamed *CG14939 Cyclin Y* (*CycY*). Outside of the cyclin domain the protein has virtually no sequence similarity to other cyclins. However, *CycY* has been highly conserved through evolution. Clear *CycY* orthologs are found in all metazoans with fully sequenced genomes, including bilaterians (e.g., insects, nematodes, vertebrates), cnidarians (e.g., the sea anenome, *Nematostella vectensis*), and the placozoan, *Trichoplax adhaerens*. Cyclin Y is also found in the choanoflagellate, *Monosiga brevicollis*, the closest known unicellular relative of metazoans, suggesting that the Y-type cyclins originated prior to the first multicellular species. Cyclin Y proteins from all of these species share substantial sequence similarity over most their length, including regions outside of the cyclin domain (Figure 2-5B). In contrast, plants, fungi, and other non-metazoan species do not have proteins with extensive sequence similarity to CycY, though they do contain the CycY-specific cyclin domain; this cyclin domain is distinct from other cyclin domains

and appears to be conserved throughout the eukaryotic kingdom (Figure 2-6). In metazoan species the level of CycY conservation is particularly high. For example, the Drosophila protein shares 52% identity with the human *CCNY* protein. This level of conservation is much higher than that observed for the cell cycle cyclins (e.g., Cyclins A, B, D, and E), which share between 20 and 41% identity between human and Drosophila (Finley et al., 1996). This suggests that CycY has an important and potentially conserved function. Surprisingly, the function of Cyclin Y has not been studied and *CycY* mutants have not been reported for any model organism.

2.3.2 Generation of a *CycY* mutant

To determine the function of Drosophila *CycY*, I set out to generate a loss-of-function mutant allele. I took advantage of the availability of a strain, d03228, bearing a P-element inserted 1,958 bp downstream of the *CycY* stop codon and 5,723 bp upstream of the start codon of the neighboring gene, *crol* (Figure 2-1). This insertion itself has no visible effect on the function of any genes in this region since the homozygous d03228 adults are completely viable and normal. I used imprecise excision to generate a small deletion around the original P-element. The deletion, E8, completely removed the *CycY* coding region while leaving the coding regions of the neighboring genes intact (Figure 2-1), as determined by PCR and sequencing (Materials and Methods). Expression of the neighboring genes, *crol* and *Pde1c*, was confirmed using RNA extracted from homozygous and heterozygous E8 second instar larvae (Figure 2-7). In contrast, *CycY* transcription was undetectable in homozygous E8 larvae. Hereafter we refer to the E8 deletion as $CycY^{E8}$.

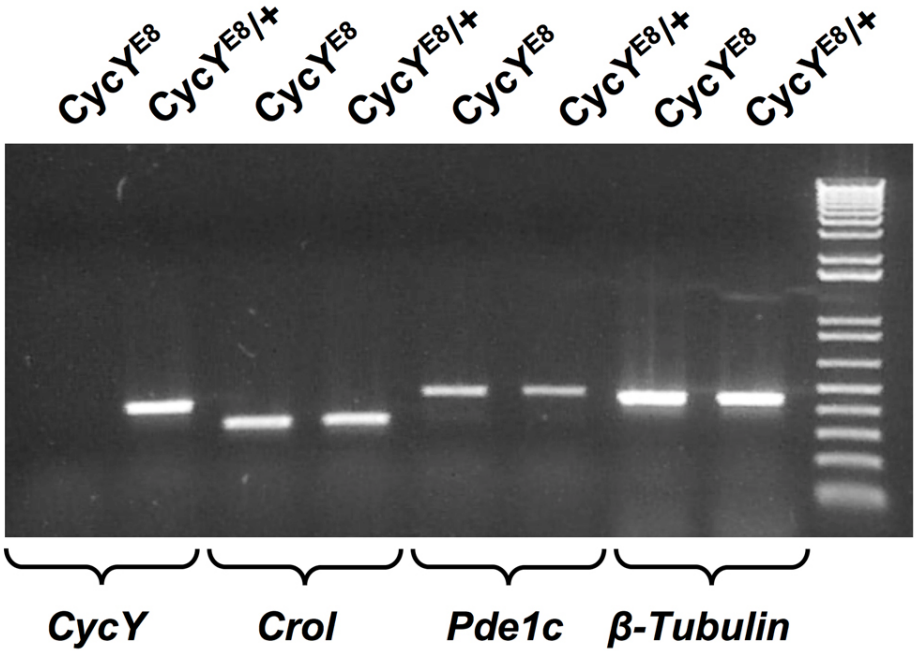


Figure 2-7. Reverse-transcription PCR detecting expression of *CycY*, *crol*, *Pde1c*, or *β-tubulin* in homozygous *CycY^{E8}* or heterozygous *CycY^{E8}* (*CycY^{E8}*/+) second instar larvae. The "+" chromosome is a *CyO* balancer with *Act5C-GFP*. A band of the expected size is detected for all genes in both genotypes. The band is missing in the homozygous *CycY^{E8}* larvae as expected.

Two additional lines of evidence indicate that *CycY* is the only gene affected in strain $CycY^{E8}$. First, $CycY^{E8}$ fully complemented $crol^{04418}$, a lethal null allele of the neighboring gene (D'Avino and Thummel, 1998); $crol^{04418}$ also complemented the mutant phenotype of $CycY^{E8}$ (see below). Thus, although $CycY^{E8}$ lacks the first non-coding exon of *crol*, a *crol* transcript is expressed and appears to be fully functional. Second, as described in detail below, all of the abnormalities that I observed in homozygous $CycY^{E8}$ mutants can be rescued either by a *CycY* genomic transgene (Figure 2-1) or by ubiquitous expression of a *CycY* cDNA using heat shock induction (see below). Combined these results indicate that the $CycY^{E8}$ mutant strain is a null mutant for *CycY*.

2.3.3 *CycY* null mutants show delayed entry into pupariation and are pupal lethal

Homozygous $CycY^{E8}$ mutants or $CycY^{E8}$ over a deficiency that removes *CycY* (*Df(2L)Exel6030*) produce no viable adults indicating that *CycY* is an essential gene. To analyze the lethal phase, eggs from a self cross of $CycY^{E8}$/*CyO* flies were collected for 12 hours and aged for another 30 hours. Of 366 embryos examined, 89 (24.3%) remained unhatched while 277 (75.7%) hatched to first instar larvae. Since roughly 25% of the embryos from this cross should be homozygous *CyO*, which is lethal during embryogenesis, a third of the embryos that hatched should be homozygous $CycY^{E8}$, indicating that zygotic expression of *CycY* is not essential for embryogenesis.

To evaluate whether *CycY* is required during larval and pupal development, I picked 180-200 first instar larvae of *CycY* null mutants (homozygous $CycY^{E8}$ or $CycY^{E8}$/ *Df(2L)Exel6030*) or their siblings and followed their morphology and development for 15

days, after which no additional adults eclosed. *CycY* null mutants did not show obvious larval lethality since the majority (90% or 93%) of first instar larvae developed into pupae, which is a rate similar to their heterozygous siblings (84% or 94%, respectively) (Table 2-4). However, I did observe delayed growth during larval development. By the time third instar larvae in the heterozygous group reached the wandering stage, *CycY* null mutant larvae were still at the feeding stage and exhibited dramatically smaller body sizes (Figure 2-8 A, B, G, H; Figure 2-9 A, B). The $CycY^{E8}$ homozygotes eventually grew to sizes that were 80-90% of the heterozygotes before pupariation (Figure 2-8 G, J). The delay in larval growth could be rescued with a genomic *CycY* transgene (Figure 2-8 C, D, I; Figure 2-9 C, D). The delay was also evident in the timing of pupariation. As shown in Figure 2-8 A and B, the first pupa of $CycY^{E8}$ heterozygotes was observed at 6 days after egg deposition (AED), while the first pupa of $CycY^{E8}$ homozygotes was observed at 7 days AED. Based on the number of pupae that formed each day in the two strains I estimated that puparium formation of $CycY^{E8}$ homozygous mutants was delayed for about 13 hours relative to that of the heterozygous controls (Materials and Methods). The genomic *CycY* transgene shortened this delay to about 5 hours. Similar results were obtained with the $CycY^{E8}$/*Df(2L)Exel6030* mutants (Figure 2-9).

CycY null mutants were arrested predominately during pupal stages, but with variable expressivity. I scored the final developmental stages of animals from each genotype based on the presence of defined morphological markers (Bainbridge and Bownes, 1981). Two major lethal phases were observed. The early lethal phase was between pupal stages P3 and P5; for example, all 162 $CycY^{E8}$ mutants that pupated developed to stage P3 but only 61% reached stage P5 (Table 2-4). In contrast, all of the

Table 2-4. *CycY* and *Eip63E* mutants display variable expressivity[a]

Genotype[b]		L1	w. L3	P1	P3	P4	P5	P14	P15	A
CycY^{E8}/+	n	180	152	152	152	152	152	151	150	150
	%	100	84	84	84	84	84	84	83	83
CycY^{E8}	n	180	162	162	162	158	110	74	23	15[c]
	%	100	90	90	90	88	61	41	13	8
CycY^{E8}/+; *P{CycY}*	n	200	180	180	180	180	180	180	180	180[d]
	%	100	90	90	90	90	90	90	90	90
CycY^{E8}; P{CycY}	n	200	185	185	185	185	185	185	177	177[e]
	%	100	93	93	93	93	93	93	89	89
CycY^{E8}/+ and *Df(2L)Exel6030/+*	n	200	187	187	187	187	187	187	174	174[f]
	%	100	94	94	94	94	94	94	87	87
CycY^{E8}/Df(2L)Exel6030	n	200	186	186	186	182	144	88	31	19[g]
	%	100	93	93	93	91	72	44	16	10
CycY^{E8}/+; *P{CycY}* and *Df(2L)Exel6030/+*; *P{CycY}*	n	200	178	178	178	178	178	178	177	177[h]
	%	100	89	89	89	89	89	89	89	89
CycY^{E8}/Df(2L)Exel6030 ; *P{CycY}*	n	200	179	179	179	179	179	179	176	176[i]
	%	100	90	90	90	90	90	90	88	88
Eip63E^{GN50}/+ and *Eip63E^{81}/+*	n	180	164	164	164	164	164	164	162	162
	%	100	91	91	91	91	91	91	90	90
Eip63E^{GN50}/Eip63E^{81}	n	180	135	129	129	76	59	0	0	0
	%	100	75	72	72	42	33	0	0	0

[a] 180 or 200 newly eclosed first instar larvae (L1) from each genotype were followed and the number that reached each stage, including wandering third instar larvae (w. L3), pupal stages (P1-P5, P14, and P15), and adults (A), was recorded.

[b] *P{CycY}* represents a genomic *CycY* transgene (Figure 1). In *CycY^{E8}*/+ and *Df(2L)Exel6030/+*, "+" stands for an *Act5C-GFP*-marked *CyO* balancer chromosome presumed to be wild type for *CycY*. In *Eip63E^{81}*/+ and *Eip63E^{GN50}*/+, "+" stands for an *Act5C-GFP*-marked *TM3, Ser* balancer chromosome presumed to be wild type for *Eip63E*.

[c] 13 out of the 15 *CycY^{E8}* adults that eclosed had leg and wing defects and died quickly, while the remaining two were much smaller than their heterozygous siblings and died within two days.

[d] 3 out of the 180 *CycY^{E8}/+; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[e] 18 out of the 177 *CycY^{E8}; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[f] One out of the 174 *CycY^{E8}*/+ and *Df(2L)Exel6030/+* adults was found dead on the food surface with the wing still folded and without other obvious morphological defects.

[g] All of the 19 *CycY^{E8}/Df(2L)Exel6030* adults that eclosed had leg and wing defects and died quickly.

[h] 6 out of the 177 *CycY^{E8}/+; P{CycY}* and *Df(2L)Exel6030/+; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

[i] 13 out of the 176 *CycY^{E8}/Df(2L)Exel6030; P{CycY}* adults were found dead on the food surface with the wing still folded and without other obvious morphological defects.

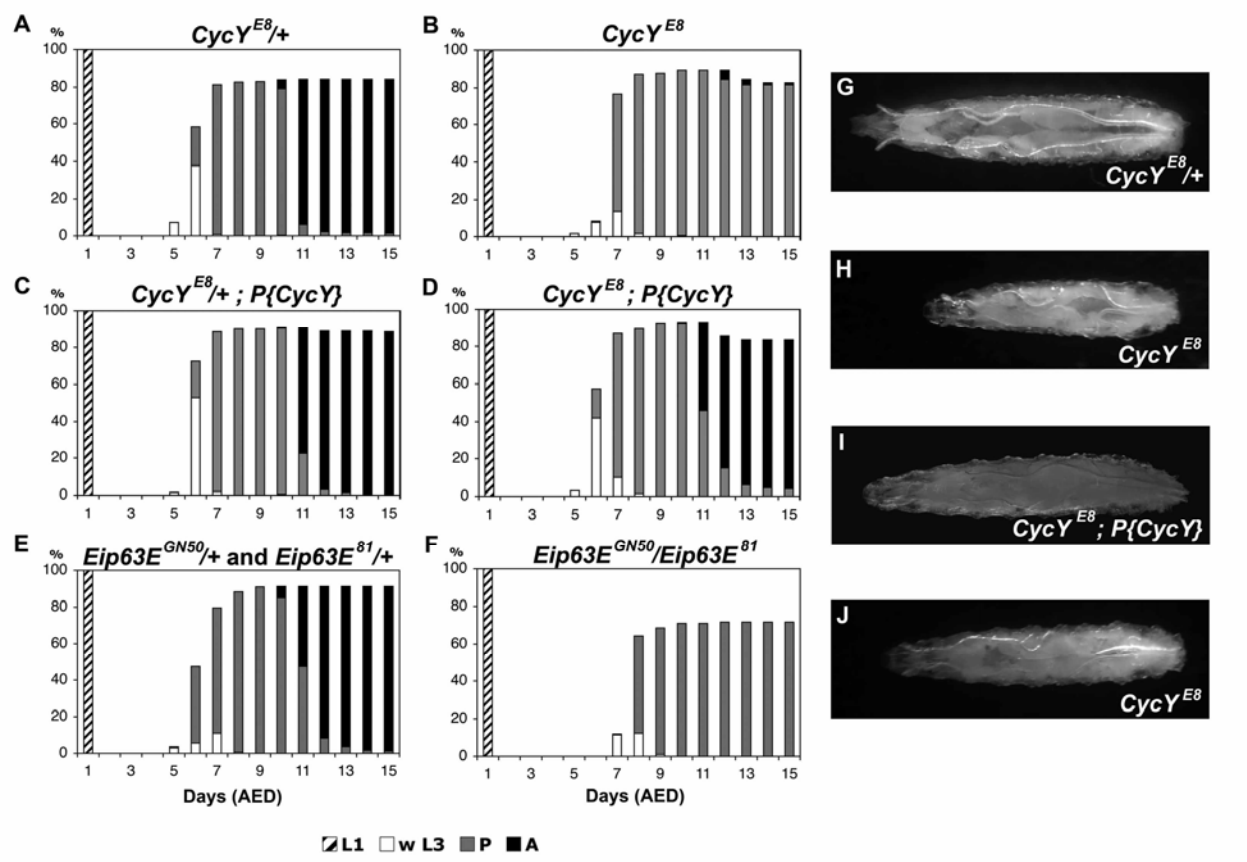


Figure 2-8. Developmental timing of *CycY* and *Eip63E* mutants. (A-F) The development of 180 first instar larvae (L1) of each genotype were followed for 15 days. Genotypes include heterozygous $CycY^{E8}$ (A and C) or homozygous $CycY^{E8}$ (B and D). Larvae in C and D harbored a genomic *CycY* transgene on the third chromosome (*P{CycY}*). Larvae heterozygous for the *Eip63E* mutants, $Eip63E^{GN50}$ or $Eip63E^{81}$ (E), or transheterozygous $Eip63E^{GN50}/Eip63E^{81}$ (F) were also analyzed. The percentage of first instar larvae that developed into wandering third instar larvae (w L3), pupae (P), and adults (A) on each day after egg deposition (AED) is shown. (G-J) Typical third instar larvae of $CycY^{E8}$/+ (G), $CycY^{E8}$ (H), $CycY^{E8}$*; P{CycY}* (I) at the same time point, and $CycY^{E8}$ after an additional day (J). In A, C, and G, "+" stands for an *Act5C-GFP*-marked *CyO* balancer chromosome; in E, "+" stands for an *Act5C-GFP*-marked *TM3, Ser* balancer chromosome.

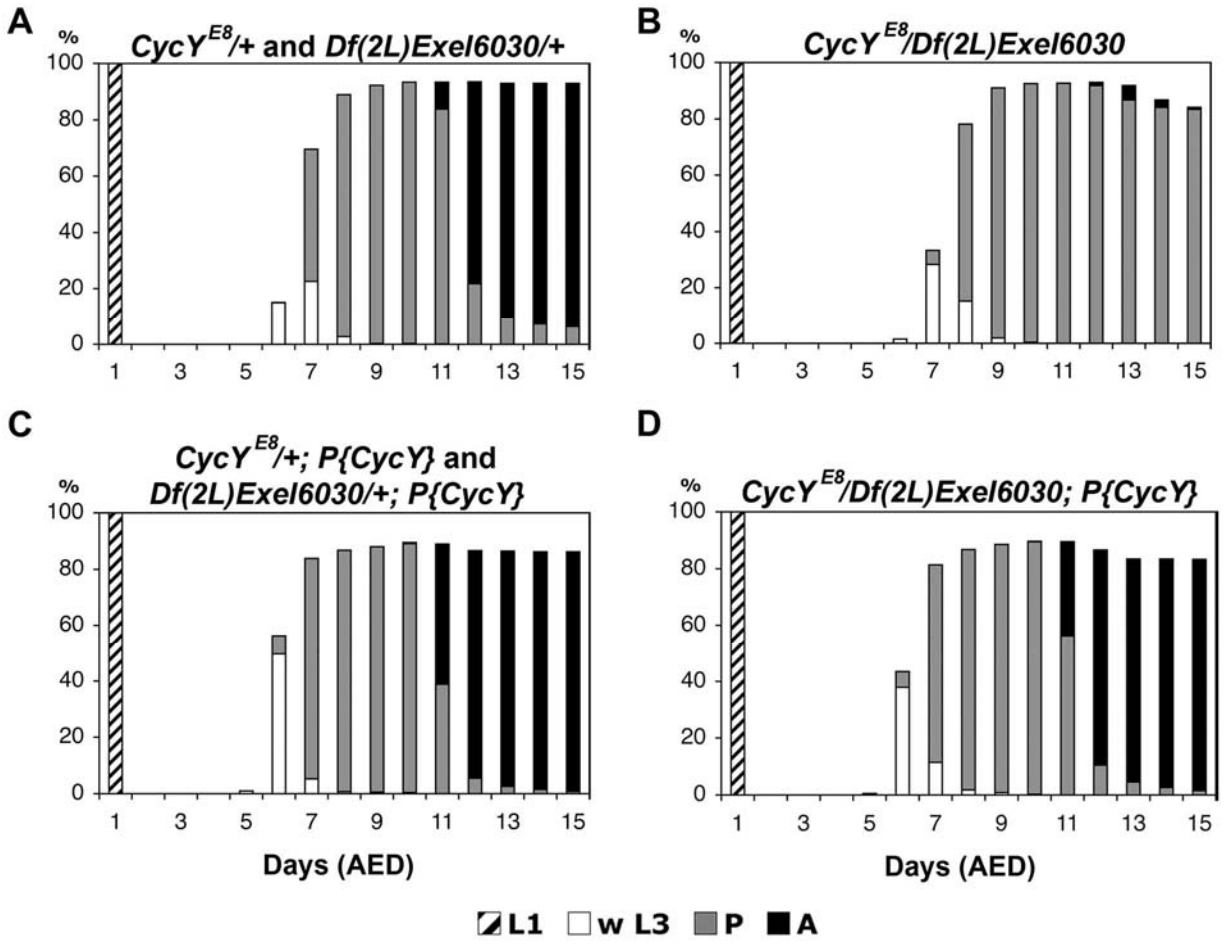


Figure 2-9. **Developmental timing of *CycY* null mutants with and without a *CycY* genomic transgene.** The development of 200 first instar larvae of each genotype was followed for 15 days. Genotypes shown include *CycY*^{E8}/+ and *Df(2L)Exel6030*/+ combined (A), *CycY*^{E8}/*Df(2L)Exel6030* (B), *CycY*^{E8}/+; *P{CycY}* and *Df(2L)Exel6030*/+; *P{CycY}* combined (C), *CycY*^{E8}/*Df(2L)Exel6030*; *P{CycY}* (D). The percentage of first instar larvae (L1) that developed into wandering third instar larvae (w L3), pupae (P), and adults (A) on each day after egg deposition (AED) is shown. "+" stands for an *Act5C-GFP*-marked *CyO* balancer chromosome presumed to be wild type for *CycY*.

152 heterozygotes that pupated reached stage P5, and all but two eventually emerged as adults. The *CycY* null pupae that were arrested at stage P3 or P4 showed a variety of developmental defects, including defects in gas bubble translocation, head eversion, leg elongation, and adult tissue growth (Figure 2-10 and Table 2-5). Many mutant individuals stopped further development with the newly formed gas bubble still in the middle of the abdomen (Figure 2-10 E). In others the gas bubble translocated to the posterior portion of the puparium as in wild-type, but then failed to completely relocate to the anterior (Figure 2-10 H), which may hinder head eversion (Chadfield and Sparrow, 1985). Many of the mutant pupae showed different amounts of empty space inside the pupal case (Figure 2-10 D-I), which was probably due either to the failure of gas bubble translocation, or to insufficient adult tissue growth. A defect in leg elongation was also prevalent. Some mutant individuals had partially elongated legs that were either shorter than normal and did not reach the bottom of the abdomen, or were bent (e.g., Figure 2-10 G). More severe cases showed no sign of leg elongation (Figure 2-10 D). Wings also did not achieve full extension. The $CycY^{E8}$/*Df(2L)Exel6030* mutant had the same range of phenotypes as homozygous $CycY^{E8}$ (Table 2-5 and Figure 2-11).

The late lethal phase of the *CycY* null was between stages P14 and P15, almost at the end of pupal development. For example, while 41% of the $CycY^{E8}$ homozygous pupae reached stage P14, only 13% reached stage P15 (Table 2-4). The P14-arrested mutants exhibited the prominent malformed leg phenotype that was also observed during earlier pupal stages (Figure 2-10 Q). In addition to the morphological defects, *CycY* null pupae were generally shorter and much lighter than wild type pupae (Table 2-6, Appendix F).

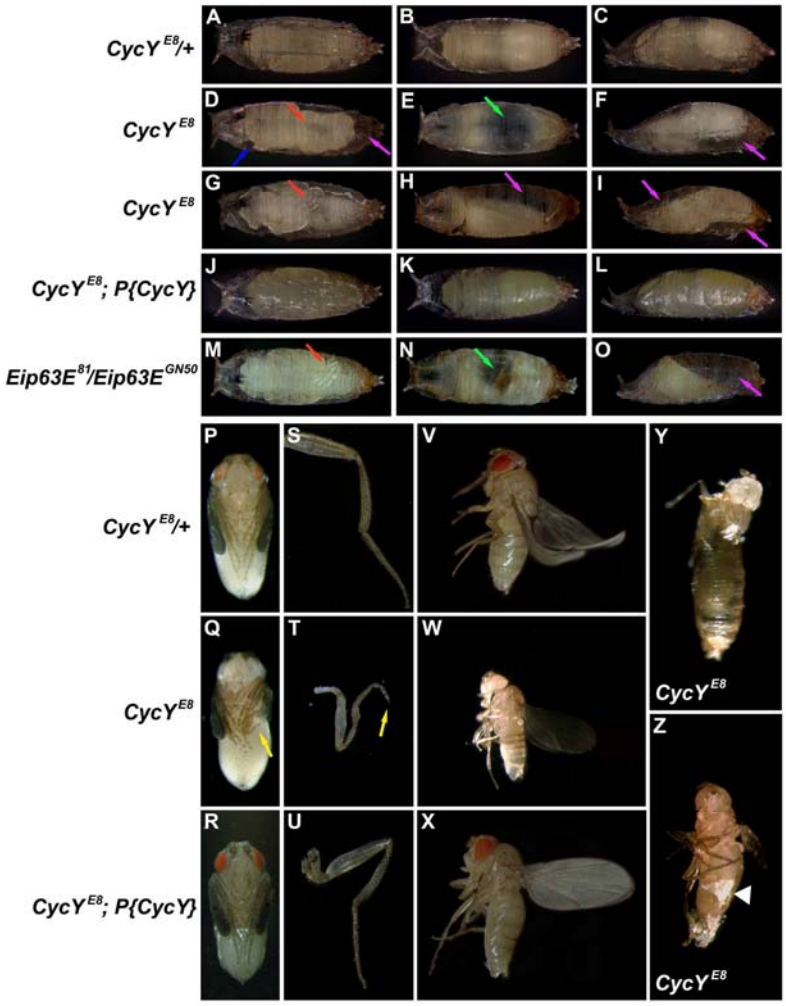


Figure 2-10. **Metamorphosis defects in *CycY* and *Eip63E* mutants.** (A-O) Representative early pupae from ventral, dorsal, and lateral views (left, middle and right columns, respectively). Genotypes include *CycY*^{E8}/+ (A-C), homozygous *CycY*^{E8} (D-I), homozygous *CycY*^{E8} with the *P{CycY}* transgene (J-L), and *Eip63E*^{81}/*Eip63E*^{GN50} (M-O). Defects are indicated by colored arrows. The *CycY*^{E8} homozygous mutant early pupae (second and third rows) and *Eip63E* null mutant early pupae (fifth row) show defects of leg elongation (red), head eversion (blue), gas bubble translocation (green), and adult tissue growth (purple). Early pupae of *CycY*^{E8} homozygotes with a genomic *CycY* transgene have no defects (fourth row). (P-R) Representative pharate adults of *CycY*^{E8}/+ (P), homozygous *CycY*^{E8} (Q), and homozygous *CycY*^{E8} with the *P{CycY}* transgene (R). Homozygous *CycY*^{E8} mutant pharate adults have an obvious bent leg phenotype (yellow arrow in (Q). *CycY*^{E8} homozygous mutant adult escapers either die soon after eclosion or survive for less than two days and have a much smaller body size (W) than heterozygous control adults (V), or *CycY*^{E8} mutants complemented with the *P{CycY}* transgene (X). Many of the adult escapers had malformed legs (T, yellow arrow), whereas legs were normal in heterozygous control adults (S), or *CycY*^{E8} mutants complemented with the *P{CycY}* transgene (U). In *CycY*^{E8} mutants arrested during eclosion (Y and Z), when the pupal case was manually removed (Z) a layer of white tissue (arrowhead) was evident. "+" stands for a *CyO* balancer chromosome with *Act5C-GFP*.

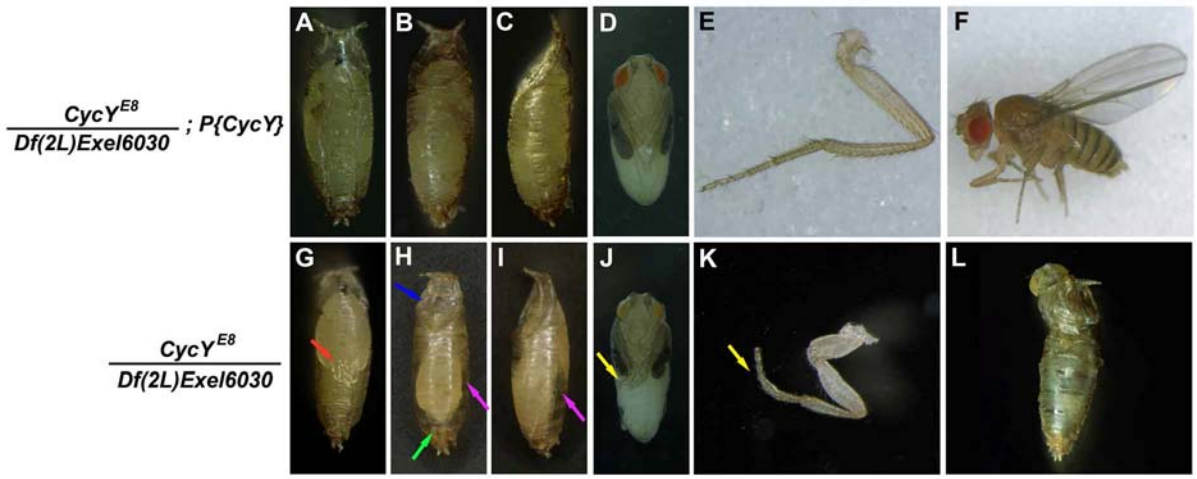


Figure 2-11. **Metamorphosis defects in *CycY* transheterozygous null mutants.** Genotypes shown include *CycY^{E8}/Df(2L)Exel6030*; *P{CycY}* (A-F) and *CycY^{E8}/Df(2L)Exel6030* (G-L). Representative early pupae (A-C, G-I), pharate adults (D, J), dissected legs (E, K), or adults (F, L) are shown. For early pupae, the first, second, and third columns present the ventral, dorsal, and lateral views, respectively. Defects are indicated by colored arrows. The *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant early pupae (G-I) show defects of leg elongation (red), head eversion (blue), gas bubble translocation (green), and adult tissue growth (purple). *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant pharate adults have an obvious bent leg phenotype (J, yellow arrow), but the dorsal view is indistinguishable from the control (data not shown). *CycY^{E8}/Df(2L)Exel6030* transheterozygous mutant adult escapers die soon after eclosion, some of which also have malformed legs (K, yellow arrow). Some were arrested during eclosion (L).

Table 2-5. Metamorphosis defects in *CycY* and *Eip63E* mutants[a]

Genotypes	Total pupae	Eclosed (%)	Arrested between P1 and P14 (%)				
			Defects	-	+	++	+++
CycY^{E8}	162	14	Leg elongation	18	9	22	37
			Empty space inside pupal case	17	16	40	13
			Head eversion	44	18	10	14
CycY^{E8}; P{CycY}	185	96	Leg elongation	4	0	0	0
			Empty space inside pupal case	4	0	0	0
			Head eversion	4	0	0	0
CycY^{E8}/Df(2L)Exel6030	186	17	Leg elongation	20	24	16	23
			Empty space inside pupal case	19	24	28	12
			Head eversion	27	35	11	10
CycY^{E8}/Df(2L)Exel6030 ;P{CycY}	179	98	Leg elongation	2	0	0	0
			Empty space inside pupal case	2	0	0	0
			Head eversion	2	0	0	0
Eip63E^{GN50}/Eip63E^{81}	129	0	Leg elongation	2	17	17	64
			Empty space inside pupal case	32	26	23	20
			Head eversion	33	23	4	40

[a] Mutants terminally arrested between pupal stages P1 and P14 (Table 2-4) were scored for metamorphosis defects (leg elongation, head eversion, or empty space inside the pupal case). - no defect; + mild defect; ++ moderate defect; +++ severe defect.

Table 2-6. *CycY* and *Eip63E* mutant pupae are smaller than wild type pupae

Genotype[c]	Average weight[a]		Average length[b]	
	mg (%)	n	% ± SD	n
CycY^{E8}/+	1.12 (100)	161	100 ± 4.0	31
CycY^{E8}/+ and *Df(2L)Exel6030*/+	1.18 (100)	239	100 ± 4.8	44
CycY^{E8}	0.65 (58)	137	90 ± 8.6	36
CycY^{E8}/Df(2L)Exel6030	0.75 (64)	230	92 ± 6.4	44
CycY^{E8}/+; P{CycY}	1.19 (100)	265	100 ± 4.9	72
CycY^{E8}; P{CycY}	1.13 (95)	239	102 ± 5.0	72
CycY^{E8}/+; P{CycY} and *Df(2L)Exel6030/+; P{CycY}*	1.21 (100)	246	100 ± 4.4	47
CycY^{E8}/Df(2L)Exel6030; P{CycY}	1.14 (94)	279	101 ± 4.2	48
Eip63E^{GN50}/+ and *Eip63E^{81}*/+	1.25 (100)	104	100 ± 3.7	30
Eip63E^{GN50}/Eip63E^{81}	0.75 (60)	142	90 ± 5.3	40

[a] Percent of average weight is calculated relative to heterozygous siblings (100%).

[b] Percent of average length is calculated relative to heterozygous siblings (100%).

[c] The plus symbol (+) stands for an *Act5C-GFP*-marked balancer chromosome; either *CyO* with *CycY^{E8}* and *Df(2L)Exel6030*, or *TM3, Ser* with *Eip63E^{GN50}* and *Eip63E^{81}*.

Among the small fraction of *CycY*^{E8} pupae that reached stage P15, 8 out of 23 (35%) arrested during the process of eclosion. The remainder eclosed into adults, but the majority (13 out of 15) died very quickly with their wings still folded. Most of these adults displayed short bent legs (Figure 2-10 T). Only two animals successfully eclosed into adults that looked normal, though they were smaller than newly emerged heterozygous control adults (Figure 2-10 V, W) and they survived for less than two days. When the mutants that were arrested during eclosion were manually dissected from the pupal case, a layer of white tissue could be seen, which seemed to adhere adult structures to the inside wall of the pupal case (Figure 2-10 Y, Z). All of the *CycY* null mutant defects described above could be rescued by introduction of a *CycY* genomic transgene (Figure 2-10 and 2-11, Table 2-4, 2-5, and 2-6).

2.3.4 The expression of CycY is essential during the transition from third instar larvae to prepupae

The null mutant phenotype of *CycY* suggested an important function during metamorphosis. To determine the developmental time point at which *CycY* expression is required, I generated transgenic flies that expressed myc-tagged CycY from a heat shock promoter. A series of different heat shock regimes were performed to compare their ability to rescue the lethality of homozygous *CycY*^{E8} (Figure 2-12). Heat shock on the first 3 days after egg laying failed to rescue the viability of homozygous *CycY*^{E8} mutants. However, when heat shock was extended for one or two more days, which included late third instar larvae, the rescue ability was dramatically increased to 30%-35%. If *CycY* was also provided during early pupal stages, the rescue ability increased

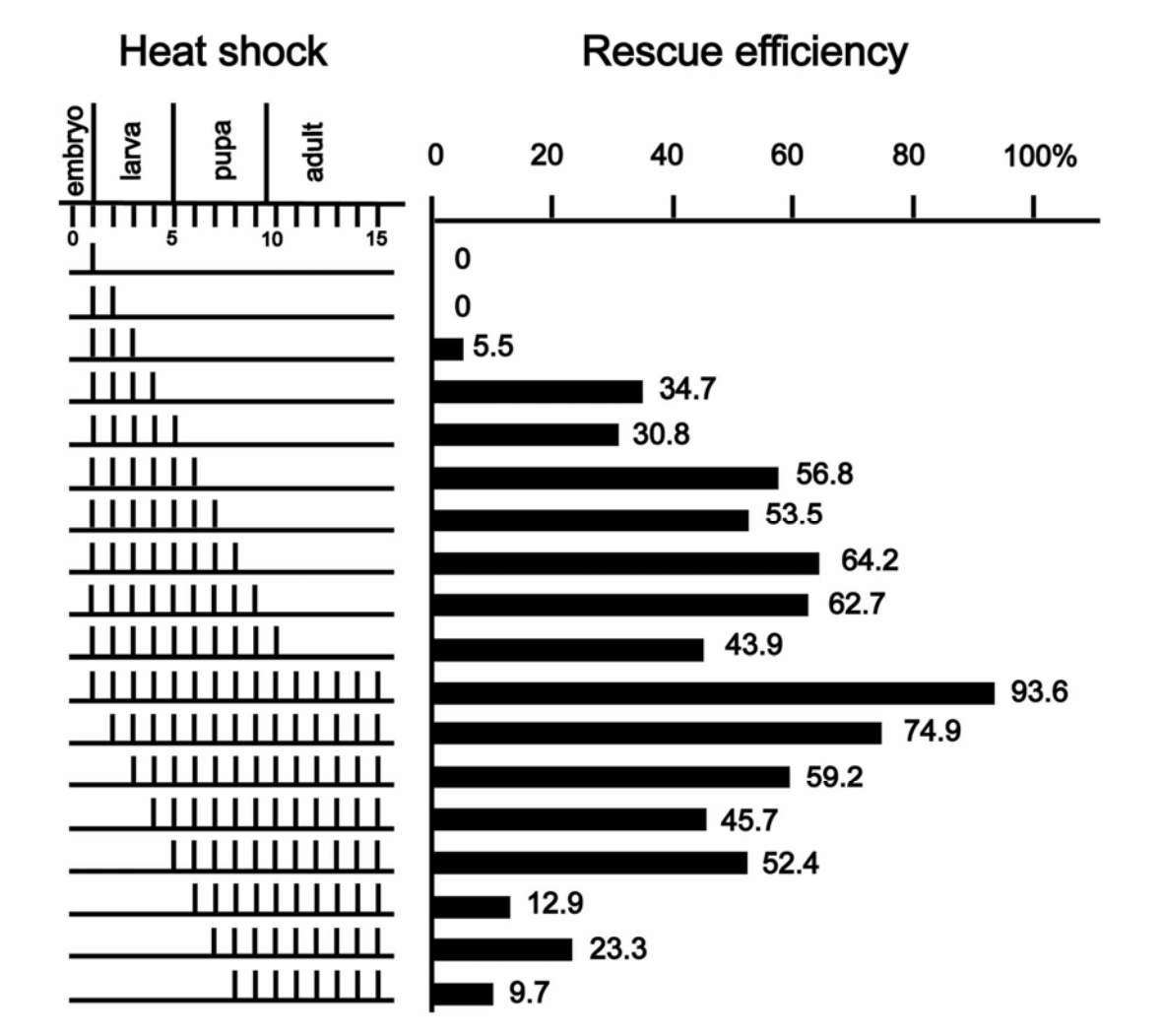


Figure 2-12. Temporal requirements for the expression of *CycY*. Embryos from a *CycY*^{E8}*/CyO; hs-CycY/TM3, Ser* self cross were collected for 24 hours and heat shocked for different regimes. Each row indicates a different heat shock schedule. On the left side, each bar represents a single 1h heat shock at 37ºC on that particular day. The efficiencies of rescue to adulthood are shown on the right. The genotype of each adult was determined by the presence or absence of *CyO* and *Ser* balancer chromosomes (Materials and Methods). Representative adult genotypes were confirmed by single-fly PCR. For each condition, the total number of adults analyzed was between 200 and 300.

further to 50-60%. If *CycY* expression was withheld until 4 days after egg-laying, a 50% rescue rate could still be achieved. However, if heat shock was delayed for one more day, the rescue ability decreased to only 13% (Figure 2-12). Combined, these data suggest that the most important period for zygotic *CycY* expression is from the late larvae to the early stages of pupal development, consistent with the first major lethal phase of the $CycY^{E8}$ mutant.

To see whether *CycY* is expressed at the developmental times when it appears to be needed, I used quantitative real-time PCR to determine the *CycY* mRNA levels. I found that the relative abundance of *CycY* mRNA fluctuated over a narrow range during development (Figure 2-13). The highest mRNA level was observed in 0-1h embryos, most likely due to maternal deposition. *CycY* message levels then decreased from later embryogenesis through the first and second instar larval stages but increased again in third instar larvae and peaked at pupal stages. The transcription variation of *CycY* is thus consistent with its essential requirement for pupariation.

2.3.5 *CycY* shows a maternal effect that can be partially rescued by zygotic expression

The mutant phenotypes described above were based on zygotic null mutants, which showed normal embryogenesis and slow but otherwise normal larval development. To test whether maternally expressed *CycY* contributes to early development I generated maternal null mutants using the ovo^{D1} dominant female sterile technique (Chou et al., 1993). *Hs-FLP/w***; CycY*^{E8} *FRT40A/ovo*^{D1} *FRT40A* females were heat shocked for 2 hours during larval development to express FLP recombinase and

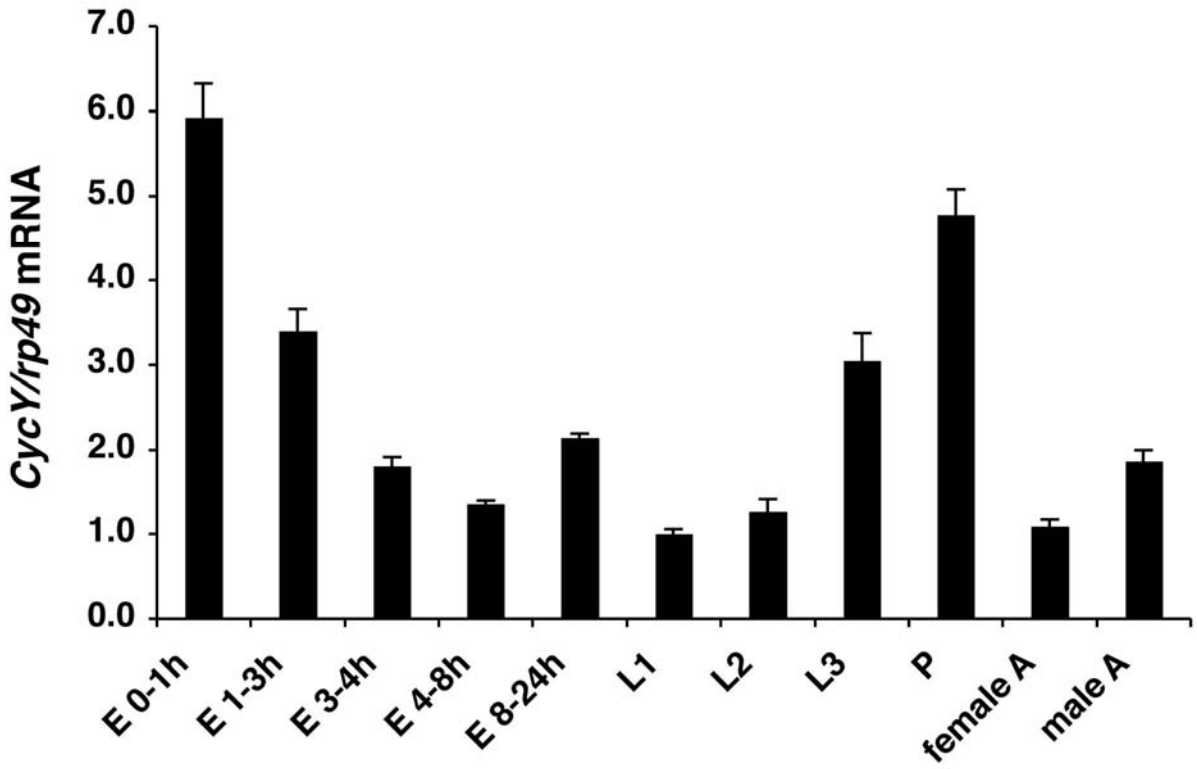


Figure 2-13. **Developmental expression pattern of *CycY*.** Total RNA was extracted from Drosophila tissues at the indicated developmental time points and mRNA levels of *CycY* were determined by quantitative real-time PCR (qPCR) as described in Materials and Methods. Expression was normalized to the mRNA levels of the internal control *rp49*.

promote homologous recombination between the *CycY^{E8} FRT40A* and *ovo^{D1} FRT40A* chromosomes. Since *ovo^{D1}* is dominant female sterile, mothers will only lay eggs if homozygous *CycY^{E8} FRT40A* germline cells are generated and *CycY* is not essential for oogenesis. Mothers that received heat shock treatment during larval development were crossed with *w^{1118}* males and the number and development of the eggs laid were monitored. I observed that heat shock treated *CycY^{E8} FRT40A/ovo^{D1} FRT40A* females could lay similar numbers of eggs as heat shock treated *FRT40A/ovo^{D1} FRT40A* females, indicating that *CycY* is not essential for at least some of the major processes of oogenesis. However, nearly 40% of the eggs from *CycY^{E8}* mothers had fused dorsal appendages or translucent body or both (Figure 2-14). These two morphological defects in some cases are common phenotypes in mutants of genes involved in axis specification (Cook et al., 2004) (G. Atikukke and R. Finley, unpublished), suggesting that *CycY* may play a role in axis specification.

To test for a maternal contribution to embryogenesis, females with homozygous *CycY^{E8}* germline cells were generated using the *ovo^{D1}* dominant female sterile technique (Chou et al., 1993), and were crossed with *CycY^{E8}/CyO, Act5C-GFP* males. Zygotic null progeny were identified by absence of the *GFP* balancer. Interestingly, the majority (99.6%) of zygotic null embryos from null mothers failed to hatch, suggesting that maternal expression of *CycY* is essential for embryogenesis. Surprisingly, when females with homozygous *CycY^{E8}* germline cells were crossed with *w^{1118}* males, 7.3% of the embryos hatched into first instar larvae and 73% of these larvae developed into normal adults. Taken together, these data suggest that maternally provided *CycY* plays

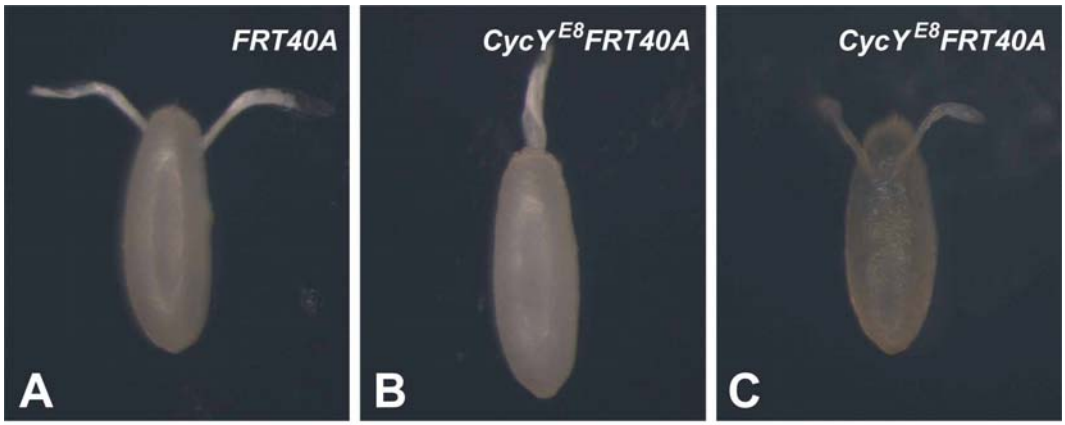


Figure 2-14. Eggs developed from homozygous *CycY^{E8}* germline cells show fused dorsal appendages or translucent body defects. A. *FRT40A*, a wild type egg showing the normal two dorsal appendages. B. *CycY^{E8} FRT40A*, egg with fused dorsal appendages. C. *CycY^{E8} FRT40A*, egg with translucent body.

an important role during embryogenesis, but that this role can be accomplished at least to a limited extent by zygotic expression.

2.3.6 Eip63E is a potential binding partner of CycY

Cyclin proteins generally serve as regulatory subunits for Cdks. In a previous high throughput yeast two-hybrid screen (Stanyon et al., 2004) we identified an interaction between CycY and Eip63E, a Cdk with no known cyclin partner (Rascle et al., 2003; Stowers et al., 2000). To test specificity, I conducted additional two-hybrid assays using additional Cdks and cyclins (Table 2-7). I found that CycY interacted only weakly or not at all with other Cdks, including Cdk1, Cdk2, Cdk4, Cdk5, Cdk7, Cdc2rk, and CG7597. Likewise, Eip63E interacted with CycY and CycC, a protein known to be promiscuous in two-hybrid assays, but only weakly or not at all with CycA, CycB, CycB3, CycD, CycE, CycG, CycH, CycJ, CycK, CycT, koko, and CG16903.

As another approach to try to identify CycY partners, I set out to do tandem affinity purification (TAP) followed by mass spectrometry (MALDI/MS-MS). I transiently expressed NTAP-CycY, which has an N-terminal protein A binding site and calmodulin binding domain, in Drosophila S2R+ cells and did a sequential protein purification by using IgG beads and calmodulin beads. Co-purified proteins were identified by MALDI/MS-MS (Materials and Methods). I did not identify a Cdk or any of the interactors that were found by yeast two-hybrid. In addition to the bait protein, NTAP-CycY, I only identified three putative CycY-associated proteins, Hsc70, Act5C, and αTub84D. All of these are abundant proteins that are among the most common nonspecific proteins often identified in co-AP/MS experiments. This result may be due to the transient

Table 2-7. CycY/Eip63E interaction specificity by yeast two-hybrid assay

	Eip63E	
	Leu2	LacZ
CycY	3	1
CycA	0	0
CycB	0	0
CycB3	0	1
CycC	3	2
CycD	0	0
CycE	0	0
CycG	0	0
CycH	0	0
CycJ	0	0
CycK	0	0
CycT	0	0
Koko	0	0
CG16903	0	0

	CycY	
	Leu2	LacZ
Eip63E	3	1
Cdk1	1	0
Cdk2	1	0
Cdk4	0.5	0
Cdk5	0.5	0
Cdk7	0	0
Cdc2rk	0.5	0
CG7597	0	0

Interactions between LexA DNA-binding domain (BD)-tagged Eip63E and activation domain (AD)-tagged cyclins (left), or AD-tagged CycY and BD-tagged Cdks (right) were tested by yeast two-hybrid mating assays. Activity for the two reporter genes, *LEU2* and *lacZ*, was scored by the growth on plates lacking leucine (scale 0-3, where 0=no growth, 3=heavy growth) and blue color on X-gal plates (scale 0-5, where 0=white, 5=dark blue).

transfection approach I used to express the bait protein. Due to the low transfection efficiency, the abundance of the bait proteins may be very high in some cells but low or absent in others. This could lead to the relatively high contamination rate observed. The establishment of stably expressing cell lines should circumvent this problem and therefore is strongly recommended for future studies. Although Hsc70, Act5C, and αTub84D are common contaminants in co-AP/MS experiments, it is of course possible that any of these proteins actually interacts with CycY. It is interesting to consider actin, which is an abundant component of the cytoskeleton. Actin has also been identified as one of the subunits of the Brahma (Brm) chromatin-remodeling complex. Its initial identification as a member of this complex was also under suspicion due to its high abundance in the cytoplasma and the lack of evidence of its nuclear localization. Several convincing experiments, however, demonstrated the existence of nuclear actin; for example, the careful isolation of nuclei from *Xenopus* oocytes to avoid contamination with cytoplasmic proteins and the development of antibody that specifically recognizes nuclear actin (G-actin instead of F-actin, which can be recognized by phalloidin) demonstrated that actin is indeed in the nucleus (Olave et al., 2002). The existence of stoichiometric amounts of actin and actin related proteins in chromatin-remodeling complexes has now been well established (Olave et al., 2002). It has been proposed that actin promotes the assembly and stability of the complex, modulates the binding of the remodeling complex to chromatin, and enhances the ATPase activity (Mohrmann and Verrijzer, 2005). Interestingly, I identified both physical and genetic interactions between CycY and the Brm chromatin-remodeling complex (discussed in Chapter 3), raising the possibility that the CycY-actin interaction is genuine.

As an alternative approach to confirm and test the specificity of the Eip63E-CycY interaction identified by yeast two-hybrid, I expressed tagged versions of Cdks and cyclins in cultured Drosophila cells and tested interaction by co-affinity purification (co-AP) followed by immunoblotting (Materials and Methods). In the co-AP assay, CycY interacted strongly with Eip63E but only weakly or not at all with Cdk2, Cdk4, or Cdc2rk (Figure 2-15 A, B). Eip63E, on the other hand, interacted much more strongly with CycY than with other cyclins tested, including CycK, CycD, and CG31232 (Koko) (Figure 2-15 C). As expected, Glycine 243 (G243) of Eip63E, which is essential for its function *in vivo* (Stowers et al., 2000), is required for binding to CycY (Figure 2-15 D). In further support of the interaction between these proteins, a recent study demonstrated an interaction between the human homolog of Eip63E, PFTK1, and human CycY using yeast two-hybrid and co-AP assays from human cells (Jiang et al., 2009). Taken together, our data and the studies with the human orthologs support the notion that CycY and Eip63E constitute a conserved cyclin-Cdk pair.

A recent large-scale phosphoproteome study in Drosophila embryos identified several phosphorylated peptides from the CycY protein (Zhai et al., 2008). A number of the phosphorylation sites are in highly conserved serine residues, suggesting that they may affect CycY function (Figure 2-5 B). One of these residues, S389, has also been found to be phosphorylated in human CycY, both in nuclear and cytoplasmic fractions (Beausoleil et al., 2004; Olsen et al., 2006). Position Ser389 in the Drosophila protein is conserved in every species that we examined (Figure 2-5 B). Moreover in one of the two preceding positions of every CycY there is another serine (S388 in Drosophila), which was also identified as a phosphorylated residue in the human protein. As a first

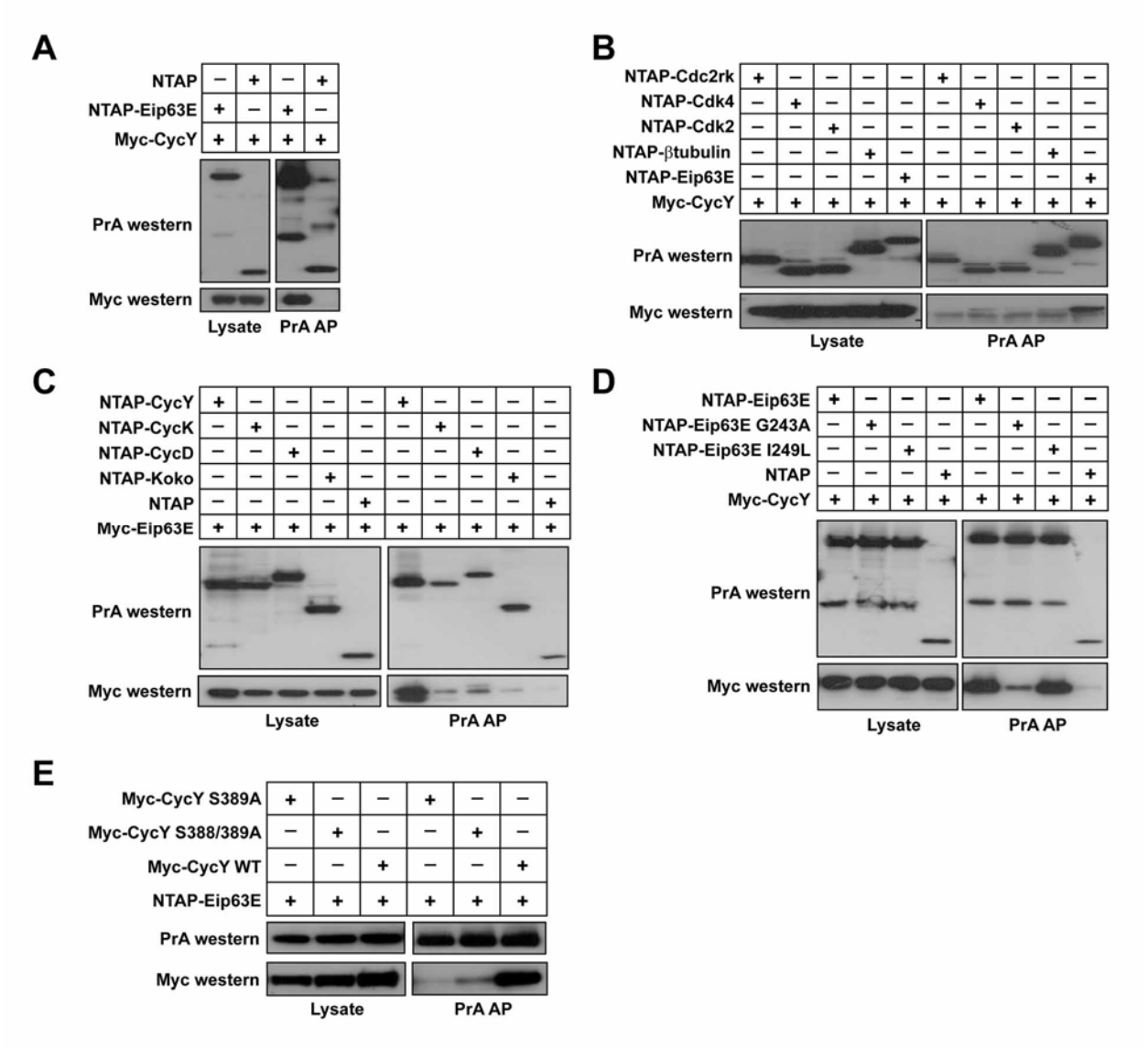


Figure 2-15. CycY preferentially interacts with Eip63E in Drosophila S2R+ cells. Cells were cotransfected with the indicated constructs and lysed for co-affinity purification (co-AP) using IgG beads. Co-purified proteins were further detected by western blot using anti-Myc or anti-protein A (PrA) antibody. (A) CycY interacts with Eip63E. (B) CycY interacts much more strongly with Eip63E than with Cdk2, Cdk4, or Cdc2rk. (C) Eip63E interacts much more strongly with CycY than with CycD, CycK, or CG31232 (Koko). (D) Eip63E G243A mutant interacts poorly with CycY. (E) CycY S389A mutants display decreased affinity for Eip63E.

test of the potential importance of these residues I generated a Drosophila CycY S389A mutant and S388A/S389A double mutant and tested their Cdk-binding ability. The Ser389A mutant had a dramatically decreased ability to bind Eip63E (Figure 2-15E). The double mutant did not further diminish Cdk binding indicating that S388 does not contribute to the interaction. While these results point to a role for S389 in Cdk interaction, I was unable to show that phosphorylation is important, since a S389E mutant also failed to interact with the Cdk (data not shown).

2.3.7 *CycY* and *Eip63E* have similar mutant phenotypes

If Eip63E and CycY form a functional Cdk/cyclin complex *in vivo*, we might expect their mutant phenotypes to be similar. Previous studies have shown that *Eip63E* is important for embryogenesis, larval development, and morphogenesis (Stowers et al., 2000). Those studies demonstrated that the majority of *Eip63E* null mutants die during larval development, while a small percentage survive to pupal stages with an occasional adult escaper. Stowers et al., also showed that puparium formation in *Eip63E* mutants is delayed by 2-3 days, pupae are small, and the rare adult escapers have a bent-leg phenotype and short life spans (Stowers et al., 2000). All of these phenotypes are similar to those I observed for $CycY^{E8}$. To further compare the *Eip63E* and *CycY* loss-of-function phenotypes, I performed a detailed side-by-side phenotypic characterization. I used a transheterozygous null mutant, $Eip63E^{81}/Eip63E^{GN50}$ (Stowers et al., 2000) and compared its phenotype with that of $CycY^{E8}$. I found that *CycY* and *Eip63E* null mutants showed similar developmental defects, though the *Eip63E* null mutant phenotype was generally more severe. Both mutants displayed a major lethal phase during

metamorphosis (Figure 2-8 A, B, E, F). While *CycY* mutants showed lethality during early or late pupal stages, the majority of *Eip63E* mutants died at earlier pupal stages (Table 2-4). Both mutants also showed similar metamorphosis defects, including gas bubble translocation defects, failed head eversion, and leg elongation defects (Figure 2-10; Table 2-5). In addition, pupae of both mutants were similarly small in weight and length (Table 2-6). Finally, both mutants exhibited delayed puparium formation, for 13 hours in the case of *CycY*, and 37 hours for *Eip63E* (Figure 2-8). I also note that Stowers et al. (Stowers et al., 2000) showed that Eip63E has a zygotically rescuable maternal contribution to embryogenesis, similar to my observation for *CycY*. The striking similarity between the mutant phenotypes of *Eip63E* and *CycY*, combined with the specific physical interaction between the proteins in yeast two-hybrid and co-AP assays, supports the idea that CycY and Eip63E may function together *in vivo*. We cannot exclude the possibility, however, that one or both proteins have additional partners. For example, one potential explanation for the earlier lethality and more severe phenotype of *Eip63E* mutants relative to the *CycY* null is that Eip63E may have functions independent of CycY and these may involve other cyclin partners. Alternatively, the subtle differences in *CycY* and *Eip63E* mutant phenotypes may be due to differences in the levels of perdurance of their maternal components. Further *in vivo* analysis of the interaction will be needed to distinguish these possibilities.

2.4 CONCLUSIONS

Cyclin Y is a highly conserved protein that has not been characterized in any model organism. Only minimal information is available for the human ortholog, *CCNY*. The gene is broadly expressed in human tissues, with particularly high levels in testis (Jiang et al., 2009; Li et al., 2009). Localization studies with *GFP* fusions in cell lines have shown that one isoform of human CycY, which has also been called CycX, is nuclear while another isoform may be anchored to the cell membrane via a conserved myristoylation signal (Jiang et al., 2009). Recently, *CCNY* was identified as a potential susceptibility factor for inflammatory bowel disease (IBD), a complicated genetic disorder affecting the intestinal mucosa. A single nucleotide polymorphism (SNP) located in an intron of *CCNY* was found to be strongly associated with the two IBD subphenotypes, Crohn's disease and ulcerative colitis (Franke et al., 2008; Weersma et al., 2009), though it is not yet clear whether *CCNY* plays a direct role in these diseases. Another study found that human CycY is among a number of proteins that are significantly upregulated in metastatic colorectal cancer cells (Ying-Tao et al., 2005), though again it is not clear whether this cyclin contributes to the phenotype of these cells. The establishment of a *CycY*-deficient animal model could provide a system for studying conserved functions of Cyclin Y and for understanding its potential role in human diseases.

Here I described the first mutant allele for a Y-type cyclin, a null for Drosophila *CycY*. I showed that *CycY* is an essential gene that is required for a broad range of developmental processes, including normal oogenesis, embryogenesis, larval and pupal development. The most obvious defects in the null were visualized during pupal

development, and included defects in gas bubble translocation, head eversion, leg elongation, and adult tissue growth. Similar phenotypes have been described for a number of genes involved in the response to the steroid hormone ecdysone, including *E74*, *EcR*, *BR-C*, and *crol* (Bender et al., 1997; D'Avino and Thummel, 1998; Fletcher and Thummel, 1995b; Kiss et al., 1988). CycY may also be involved in the ecdysone response. Consistent with this possibility, I provide several lines of evidence suggesting that at least one of the Cdk partners for CycY is the ecdysone-inducible protein, Eip63E. CycY and Eip63E preferentially interact in yeast two-hybrid assays and in co-AP assays from cultured Drosophila cells. The human orthologs of these proteins have also been shown to interact and to colocalize in human cell lines (Jiang et al., 2009). Finally, the mutations in *Eip63E* and *CycY* show a similar range of phenotypes. Our findings in Drosophila should provide a model system for further biochemical and genetic studies on the function of this conserved Cdk/cyclin pair.

CHAPTER 3

CYCLIN Y GENETICALLY INTERACTS WITH BRM COMPLEX COMPONENTS IN DROSOPHILA

3.1 INTRODUCTION

CycY is a highly conserved protein found in all metazoans. I previously showed that *CycY* is an essential gene that is required for many developmental processes (Liu and Finley Jr, 2010). A null mutant of *CycY* is lethal and exhibits delayed larval growth and major developmental defects during metamorphosis. Analysis of germline mutant clones also revealed a role of *CycY* during oogenesis and embryogenesis. CycY interacts specifically with the Cdk, Eip63E, both in yeast two-hybrid assays and in co-AP assays in cultured Drosophila cells (Liu and Finley Jr, 2010; Stanyon et al., 2004). The striking phenotypic similarity between *CycY* and *Eip63E* null mutants and the physical interaction between CycY and Eip63E supports the notion that CycY and Eip63E constitute a conserved cyclin-Cdk pair. This conclusion was further supported by several recent studies with human cell lines, in which the orthologs of CycY and Eip63E, which is generically known as Cdk14, were shown to interact and co-localize to the plasma membrane (Davidson et al., 2009; Jiang et al., 2009). While the Drosophila null phenotypes of *CycY* and *Eip63E* indicate that they are essential for development, they have not provided clues about potential tissue-specific or cell-specific functions of the CycY/Eip63E complex. A recent study found that both CycY and Eip63E are required for maximal phosphorylation of the Wnt co-receptor, LRP6, and for maximal Wnt signaling in cultured cells (Davidson et al., 2009). This led to a model in which

membrane-associated CycY recruits Cdk14 to the membrane where it phosphorylates LRP6 to help prime LRP6 for activation by Wnt. However, it is not clear whether or not CycY plays a role in Wnt signaling *in vivo* in Drosophila. The null phenotypes of *CycY* and *Eip63E* are not typical of *wingless* pathway mutants. This suggests that CycY may belong to other important pathways instead of or in addition to the Wnt signaling pathway. Here I set out to find pathways to which CycY may belong by testing for tissue- or cell-specific requirements for CycY and screening for genetic interactions with candidate pathway members.

The early pupal lethality of the *CycY* null makes it difficult to study the gene's potential role in later stages or in particular tissues. This difficulty can be overcome by conditionally knocking down gene expression in a spatially and temporally controlled manner by regulated synthesis of double-stranded RNAs (dsRNAs). dsRNA induces gene-specific silencing, a highly conserved phenomenon known as RNA interference (RNAi) (Fire, 1999; Fire et al., 1998). In Drosophila, long dsRNAs can be used as a tool to knock down expression of specific genes because Drosophila lacks the interferon-mediated immune response, which shuts down global protein synthesis and promotes general mRNA degradation in mammalian cells (Echeverri and Perrimon, 2006). Long dsRNAs expressed in or introduced into Drosophila cells are cleaved by the enzyme Dicer into short interfering RNAs (siRNAs), which are then incorporated into the RNA-induced silencing complex (RISC) to induce target mRNA degradation. Tissue-specific gene knockdown can be achieved by expressing the dsRNA using the Gal4/UAS system (Brand and Perrimon, 1993). In this system, dsRNA expression is put under control of the Upstream Activating Sequence (UAS) form yeast, which harbors binding

sites for the Gal4 transcription factor. Transgenic flies containing the UAS-dsRNA gene can be crossed with any of a variety of available lines that express Gal4 in specific tissues. In the progeny, dsRNAs will be generated only in the tissues where Gal4 is expressed. Here I used the Gal4/UAS system and dsRNA directed at *CycY* to knock down *CycY* expression in specific tissues to reveal a role for the gene in wing development.

CycY has been identified to physically interact with Snr1 in a high throughput yeast two-hybrid screen (Giot et al., 2003). This prompted us to investigate the relationship between CycY and Snr1, and to test whether Snr1 is a downstream target of CycY/Eip63E. Snr1 is a core subunit of the Brm chromatin-remodeling complex in Drosophila, which is involved in regulation of transcription. There are two subclasses of Brm complex, BAP and PBAP, which differ in their subunit structure and function (Moshkin et al., 2007). Snr1 is a member of both complexes, as are Brm, Mor, actin, BAP55 (actin related protein), BAP60, BAP111, and possibly BAP74 (Hsc70-4) (Kal et al., 2000; Mollaaghababa et al., 2001; Papoulas et al., 1998). BAP and PBAP complexes also have distinct accessory components called signature components (Chalkley et al., 2008; Collins et al., 1999; Kaeser et al., 2008; Mohrmann et al., 2004). The core subunits provide the basic structure and enzymatic activity of the complex, while the signature components provide distinct functional specificity (Moshkin et al., 2007). BAP and PBAP function coordinately, independently, or antagonistically on target gene transcription and modulate distinct biological processes. For example, the well-known functional requirement of the Brm complex for entry into mitosis is believed to be executed by BAP, but not PBAP (Moshkin et al., 2007). An important cell cycle

regulator, stg (cdc25 phosphatase), which triggers mitosis, was down regulated in cultured Drosophila cells in which BAP components were knocked down by RNAi, and in *Snr1* temperature-sensitive mutants (Moshkin et al., 2007; Zraly et al., 2004). A direct interaction between BAP and the *stg* promoter region has also been demonstrated by chromatin immunoprecipitation (ChIP) (Moshkin et al., 2007).

By modulating chromatin structures, the Brm complex is involved in both gene activation and suppression. Several interesting groups of genes have been identified to be the direct targets of Brm complex regulation. These include the Hox genes, a group of related genes that specify the anterior-posterior axis and determine the segment identity during early embryonic development. Hox genes include the Antennapedia complex (ANT-C) and the bithorax complex (BX-C), which encode homeodomain transcription factors (Duncan, 1987; Kaufman et al., 1990). The transcription of Hox genes is ultimately sustained in the off or on state in late embryogenesis by the action of the Polycomb group (PcG) of repressors and the trithorax group (trxG) of activators, respectively (Harding and Levine, 1988; Ingham, 1988; Orlando and Paro, 1995). Brm was initially identified as a member of the trithorax group that dominantly suppressed Polycomb (Pc) mutations (Kennison and Tamkun, 1988). BAP, the osa-containing Brm complex, represses transcription of Wg target genes, such as *nub* and *dpp* (Collins and Treisman, 2000). BAP is also required for activation of several targets of EGFR signaling involved in wing vein development, such as *Delta*, *rhomboid*, and *argos* (Marenda et al., 2004; Terriente-Felix and de Celis, 2009), though it is still unclear if the complex regulates these genes directly. Finally, a cluster of Ecdysone-induced genes (*Eig*) were found to be strongly misregulated in *Brm* and *Snr1* mutants and these genes

were further shown to be the direct targets regulated by the Brm complex in cultured cells (Zraly et al., 2006). This established a direct connection between chromatin structure modification and ecdysone signaling.

The core subunit of the Brm complex, Brm ATPase, is expressed at all developmental stages with a relatively high level throughout embryogenesis and in pupae (Elfring et al., 1998). In addition to the ATPase domain, Brm proteins possess a Bromodomain, which is ~ 110 amino acids and specifically recognizes acetylated lysine in the histone tail (Mohrmann and Verrijzer, 2005). Surprisingly, deletion of the Bromodomain does not affect chromatin binding and appears to be dispensable for Brm function. On the other hand, domain II, which is located N-terminal to the ATPase domain, contributes essential functions to the assembly or stability of the Brm complex (Elfring et al., 1998). Lysine (K) 804 is a conserved amino acid in the ATP-binding site. Mutation of this lysine to arginine (R) eliminates enzymatic activity but conserves the protein's ability to assemble into the 2 megadalton (MD) Brm complex. This mutant is used widely as a dominant negative antimorphic allele of *Brm* for functional studies. By analyzing animals ectopically expressing Brm^{K804R}, or mosaic animals created with a *Brm* null allele, Brm has been shown to be important for the development of the peripheral nervous system, preventing homeotic transformations, and cell viability (Elfring et al., 1998).

Snr1 has been suggested to function as a regulatory subunit by recruiting other transcription factors to constrain Brm complex activity in particular tissues (Marenda et al., 2004). The spatial and temporal expression pattern of *Snr1* is similar to that of *Brm* (Dingwall et al., 1995), except for a few differences (Zraly et al., 2003). One striking

example is that Snr1 is barely detected in leg imaginal discs where Brm is highly expressed, suggesting that Snr1 only functions in a subset of Brm complexes (Zraly et al., 2003). *Snr1* homozygous zygotic null mutants die before entering the third instar larval stage (Dingwall et al., 1995), while germline clone analysis revealed its essential function during oogenesis (Zraly et al., 2003). Mosaic animals with somatic null clones showed similar phenotypes to *Brm* mutants, such as reduced cell viability and peripheral nervous system defects. However, consistent with the expression differences between *Snr1* and *Brm*, clones of a *Snr1* null revealed no function during leg development (Zraly et al., 2003). Surprisingly, Snr1 is required for adult viability, which has not yet been linked with Brm in Drosophila (Marenda et al., 2003; Zraly et al., 2003). However, in human adrenal cortex carcinoma derived cell line SW13, Brg1 could induce a senescent-like morphology (Shanahan et al., 1999). In addition, Brm1 levels increase in the liver of old mice (Iakova et al., 2003). Not only ATP-dependent chromatin remodeling, but other chromatin modifications, such as histone acetylation, deacetylation, DNA methylation, have also been suggested in organismal aging (Bandyopadhyay and Medrano, 2003).

Drosophila Snr1 and its orthologs in yeast (SNF5) and human (hSNF5/INI1) share a highly conserved domain, including two direct repeats and a coiled-coil (CC) region. The repeat domain in Snr1 and INI1 has been shown to mediate protein-protein interactions to direct the metazoan SWI/SNF complex to target loci (Cheng et al., 1999; Kalpana et al., 1994; Rozenblatt-Rosen et al., 1998). Contrary to the nuclear localization of wild type, a truncated Snr1 mutant, Snr1-2, which lacks the CC domain and part of repeat 2, is predominately localized in the cytoplasm, probably due to the exposure of

the nuclear export signal (NES, 248-261) immediately N-terminal to the truncation site (Brumby et al., 2002; Zraly et al., 2003). A small fraction of *Snr1-2* mutant protein still localizes to the nucleus and assembles into the Brm complex (Zraly et al., 2003). Since *Snr1-2* phenotypes are sensitive to *Snr1* dosage, *Snr1-2* has been used as a dominant negative allele of *Snr1*. Ectopic expression of *Snr1-2* led to several striking developmental defects including extra wing vein along L2, incomplete abdominal tergite fusion along the dorsal midline, and decreased adult viability (Zraly et al., 2003).

In this chapter, I describe the consequences of conditionally knocking down *CycY* expression in specific tissues. I show that *CycY* is required for wing growth and sustained adult viability. I also show that *CycY* genetically interacts with *Snr1* and *Brm*, two components of the Brm ATP-dependent chromatin-remodeling complexes, and that the *CycY* protein can physically interact with Snr1. Furthermore, I show that the downstream targets of Brm complexes, *Eig71Eh* and *Eig71Ei*, are misregulated in *CycY* mutants. Taken together, these data suggest that *CycY* may be involved in gene regulation by modulating Brm complex activity.

3.2 MATERIALS AND METHODS

3.2.1 Fly stocks

All fly stocks were maintained in vials containing standard cornmeal molasses medium. *GMR-Gal4*, *en-Gal4*, *69B-Gal4*, *e22c-Gal4,* and *Act5C-Gal4* were obtained from the Bloomington stock center (stock numbers 1104, 6356, 1774, 1973 and 4414 respectively). *UAS-Snr1-2, Snr1*^{R3} (Zraly et al., 2003) was kindly provided by Dr. Andrew K. Dingwall. *UAS-Brm*^{K804R} (Elfring et al., 1998) was kindly provided by Dr. Jessica E.Treisman. *y*^{1}*w** *hs-FLP; Ubi-GFP FRT40A* was kindly provided by Dr. Dongbin Xu. The UAS-RNAi lines, *UAS-dicer2*, *UAS-Snr1i*, and *UAS-Brmi*, were obtained from the Vienna Drosophila Resource Center (transformant IDs 60008, 12644, and 37720 respectively). *CycY*^{E8}, *CycY*^{E8} *FRT40A, UAS-CycY*, and *hs-CycY* have been described previously (Liu and Finley Jr, 2010). All fly strains used in this study are listed in Appendix G.

3.2.2 Plasmid cloning for P-element transformation

pWIZ-CycYi^{N} was constructed by first subcloning an XbaI/XbaI fragment which included the 5' 605bp of the *CycY* cDNA, beginning with the ATG, into the NheI site of pWIZ (Lee and Carthew, 2003) in the sense orientation to make pWIZ-CycYi^{N}-sense. This XbaI/XbaI fragment was generated by PCR from pAS1-CycY (Finley lab # 897) using oligonucleotides [forward: 5' ATGCTCTAGAATGGGCAACAAGAACTCG (Finley lab # 679); reverse: 5' ATGTCCTCTAGACGATCCGATTGCCGATTC (Finley lab # 680)], which provided XbaI digestion site at both ends. Next, an XbaI/EcoRI fragment

containing the same 5' 605bp of the *CycY* cDNA, beginning with the ATG, was subcloned into the AvrII/EcoRI site of pWIZ-CycYi^{N}-sense in the anti-sense orientation to make pWIZ-CycYi^{N}. This XbaI/EcoRI fragment was generated by PCR from pAS1-CycY (Finley lab # 897) using oligonucleotides [forward: 5' ATGCTCTAGAATGGGCAA CAAGAACTCG (Finley lab # 679); reverse: 5' ATGTCCGAATTCCGATCCGATTGCCG ATTC (Finley lab # 681)], which provided XbaI and EcoRI digestion site at each end respectively. pWIZ-CycYi^{C} was constructed similarly, by subcloning the 3' 616bp of the *CycY* cDNA ending with the stop codon in both orientations into pWIZ. In this case, the XbaI/XbaI fragment was generated by PCR from pAS1-CycY (Finley lab # 897) using oligonucleotides [forward: 5' GCAATCTCTAGACCGGCTGGACATCTTCGACG (Finley lab # 682); reverse: 5' GCTTGGTCTAGATCACGATAGTATGGCCACG (Finley lab # 683)], which provided XbaI digestion site at both ends. The XbaI/EcoRI fragment was generated by PCR from pAS1-CycY (Finley lab # 897) using oligonucleotides [forward: 5' GCAATCTCTAGACCGGCTGGACATCTTCGACG (Finley lab # 682); reverse: 5' GCTTGGGAATTCTCACGATAGTATGGCCACG (Finley lab # 684)], which provided XbaI and EcoRI digestion site at each end respectively. P-element mediated transformation was performed as previously described (Rubin and Spradling, 1982). Plasmids used or constructed for this study are listed in Appendix H. All transgenic lines generated for this study are listed in Appendix I.

3.2.3 Analysis of the wing phenotype

Wings were dissected from adult flies and mounted for microscopic examination in mineral oil. Pictures were taken with Leitz fluorescence microscope and SPOT RT3

camera. The entire wing was photographed with 100x magnification and a close-up of defined area of the wing was photographed with 630x magnification. The sizes of the posterior and anterior areas of the wing were independently measured using Image J software (Collins, 2007) and the ratio of the posterior to anterior areas (P/A) was calculated. For each genotype analyzed, twenty wings were scored. To count cell numbers, defined areas of the posterior and anterior compartments of the wing, as indicated in Figure 3-2A, were photographed and the number of bristles in the picture was counted; the number of bristles was taken as the number of cells in each area since each wing cell secret only one bristle during development (Meyer et al., 2000). Error bars indicate standard deviation of 20 individual measures. The ratio of posterior cell size to anterior cell size is equal to the number of cells in the defined area in the anterior region divided by that in the posterior region. The ratio of the posterior cell number to anterior cell number is equal to the ratio of the posterior to anterior areas divided by the ratio of the posterior to anterior cell sizes.

3.2.4 Immunostaining

Wing imaginal discs from third instar larvae were dissected in 1 x PBS and put quickly on ice. Discs were fixed in freshly made 4% paraformaldehyde in 1 x PBS at room temperature for 30 minutes and washed with PBS 3 times. Discs were then incubated with primary antibody at 4°C overnight followed by 2-3 hours incubation at room temperature with secondary antibody. The following antibodies were used: rabbit anti-GFP (1:50; Invitrogen); rabbit polyclonal anti-Histone H3 phospho-Ser10 (1:50; Upstate Biotechnology); FITC-conjugated goat anti-rabbit IgG (1:200; Jackson

Immunoresearch); Texas Red goat anti-rabbit IgG (1:200; Invitrogen); Alexa Fluor® 488 goat anti-rabbit (1:200; Invitrogen). DAPI (1μg/ml; Sigma) was used to counterstain the DNA. TUNEL staining was performed as previously described with minor modification (Wang et al., 1999). Briefly, after secondary antibody incubation, discs were blocked in block buffer (50mM Tris.HCl pH6.8, 150mM NaCl, 0.5% NP-40, 5mg/ml BSA) at 4°C overnight and then incubated in 100mM Na-Citrate, 0.1% TritonX-100 at 65°C in a water bath for 30 minutes. After three quick washes in wash buffer (50mM Tris.HCl pH6.8, 150mM NaCl, 0.5% NP-40, 1mg/ml BSA), discs were incubated in TUNEL dilution buffer (Roche) twice for 5 minutes each time at room temperature. After 30 minutes incubation in 50μl of labeling solution (*in situ* cell death detection TMR Red kit, Roche) at 37°C in a water bath, 5μl of Terminal deoxynucleotidyl transferase (TdT) enzyme solution was added and discs were incubated for 2 more hours. Discs were washed in wash buffer and mounted in Vectashield (Vector laboratories) for imaging.

3.2.5 Analysis of adult lifespan

For each genotype analyzed, males and females were collected within 24 hours of eclosion and were maintained in vials with fresh cornmeal molasses medium at the indicated temperature. Each vial contained either 30 males or 30 females. A total of 150 adults were analyzed for each genotype and sex. Adults were transferred to fresh vials every 1-2 days for 25 to 50 days and the number of dead adults was counted after each transfer. For analyzing the adult lifespan of the $CycY^{E8}$ homozygous mutant, since it is lethal during metamorphosis, I rescued mutant animals to adults by expressing a *CycY* cDNA under control of a heat-shock promoter (Liu and Finley Jr, 2010). Heat shock was

applied once every day at 37°C in a water bath from one day after egg laying until adult eclosion, and then the adults were kept at 25°C. *CycY*^{E8} heterozygous control animals were similarly treated with heat shock although the exogenous CycY is not required for these animals to reach the adult stage. To test whether the adult requirement of *CycY* can be rescued by providing exogenous CycY, in a separate experiment, these animals were continuously applied heat shock treatment once everyday from one day after egg laying until 35 days after adult eclosion.

3.2.6 Gene expression

Gene expression was assayed by reverse-transcription and quantitative real-time PCR (RT-qPCR) as previously described (Liu and Finley Jr, 2010). First instar larvae with the desired genotypes were collected and transferred to standard fly medium. Newly formed white prepupae (0 hour prepupae) were collected and transferred to a petri dish with a piece of wet filter paper and allowed to age at 25°C for the times indicated in Figure 3-9 (Fletcher and Thummel, 1995a). About 30 pupae were collected at each time point for RNA isolation. qPCR reactions were carried out in triplicate for each RNA sample. The primers used in this work are listed in Table 3-1. *rp49* was used as the internal control gene and the mRNA level of each analyzed gene was normalized to *rp49* levels.

3.2.7 Co-affinity purification (co-AP) assays

Co-AP assays were conducted as previously described (Liu and Finley Jr, 2010).

Table 3-1. Primers used for qPCR in this study

Gene	Primers	Sequence	Position[a]	Product length (bp)
Eig71Eh	DL147	5'- GTTGACTGTCTGCTTCCTGGTGAT	41-64	230
	DL165	5'- TTGCTCGATTCGGAGAAGCTATCG	247-270	
Eig71Ei	DL149	5'- CTGCCATTAGCTATTGTGTGCCTG	51-74	202
	DL150	5'- ATCGGCTAACATCTGACCATCCAG	229-252	
Eig71Eg	DL172	5'- TTGATGTGCCAGGTCCTAACTCAG	77-100	261
	DL173	5'- TTATTAAGACCGCAGGCTATCGGC	314-337	
Eig71Ef	DL174	5'- CTGCCGAAAGTTAAGGGACACTTG	116-139	235
	DL175	5'- GGCATTCTTCTAAGTTCGCCTTGG	327-350	
EcR	DL176	5'- TCAGGCGTATAATGAGTCAACCCG	1624-1647	345
	DL177	5'- CCACCTTCATCGAGAACATTTGGC	1945-1968	
Eip93F	DL155	5'- CAACCATCGGAACAATGACTACGC	3398-3421	396
	DL156	5'- GATTCGCACTTGATGTGACTGCTG	3770-3793	
ImpE2	DL170	5'- GGCCACTGAAATCAAAGAGGAACC	531-554	234
	DL171	5'- CCAATTGGCGAGTTCTGGATCAAC	741-764	
Eip63E	DL168	5'- GTCGCAATACATGGAGAAGCATCC	1007-1030	367
	DL169	5'- GTAGGTATCACGTATGCCCGGAAA	1350-1373	
rp49	DL118	5'-GATATGCTAAGCTGTCGCACAAATGGC	95-121	118
	DL119	5'-GTGCGCTTGTTCGATCCGTAACCG	189-212	

[a] Inclusive nucleotide positions in predicted transcript RA for each gene.

3.3 RESULTS

3.3.1 Generation of *CycY* conditional knockdown transgenic flies

I set out to generate transgenic Drosophila that express long double-stranded RNA (dsRNA) to knock down the expression of *CycY* in a temporally and spatially controlled manner using the UAS/Gal4 system (Brand and Perrimon, 1993; Lee and Carthew, 2003). To avoid misinterpreting gene knockdown phenotypes that may arise from off-target effects (Dietzl et al., 2007; Kulkarni et al., 2006; Ma et al., 2006; Ni et al., 2009), I generated two nonoverlapping *CycY* RNAi constructs. One (UAS-*CycYi*^{N}) targeted the N-terminal coding region and the other (UAS-*CycYi*^{C}) targeted the C-terminal coding region (Figure 3-1A). To test the knockdown efficiency, Drosophila S2R+ cells were cotransfected with each RNAi construct along with a construct that expresses NTAP-tagged CycY. Both the N-terminal and C-terminal RNAi constructs efficiently knocked down the ectopically expressed CycY, while the control RNAi (targeting *Koko*) had no effect (Figure 3-1B). I generated multiple transgenic fly strains containing each *CycYi* construct and tested the gene knockdown effect in different tissues using different Gal4 driver lines. Ubiquitous expression of both *CycYi* constructs using *Act5C-Gal4* induced lethality. *CycYi*^{N} knockdown flies did not survive beyond second instar larval stage, which is an earlier lethal phase than *CycY*^{E8} null mutants, most of which survive to pupal stages (Liu and Finley Jr, 2010). Ubiquitous knockdown with *CycYi*^{C} showed a lethal phase and morphological defects similar to *CycY*^{E8} null mutants (Liu and Finley Jr, 2010). Both RNAi constructs result in similar levels of transcript knockdown in second instar larvae (Figure 3-1C).

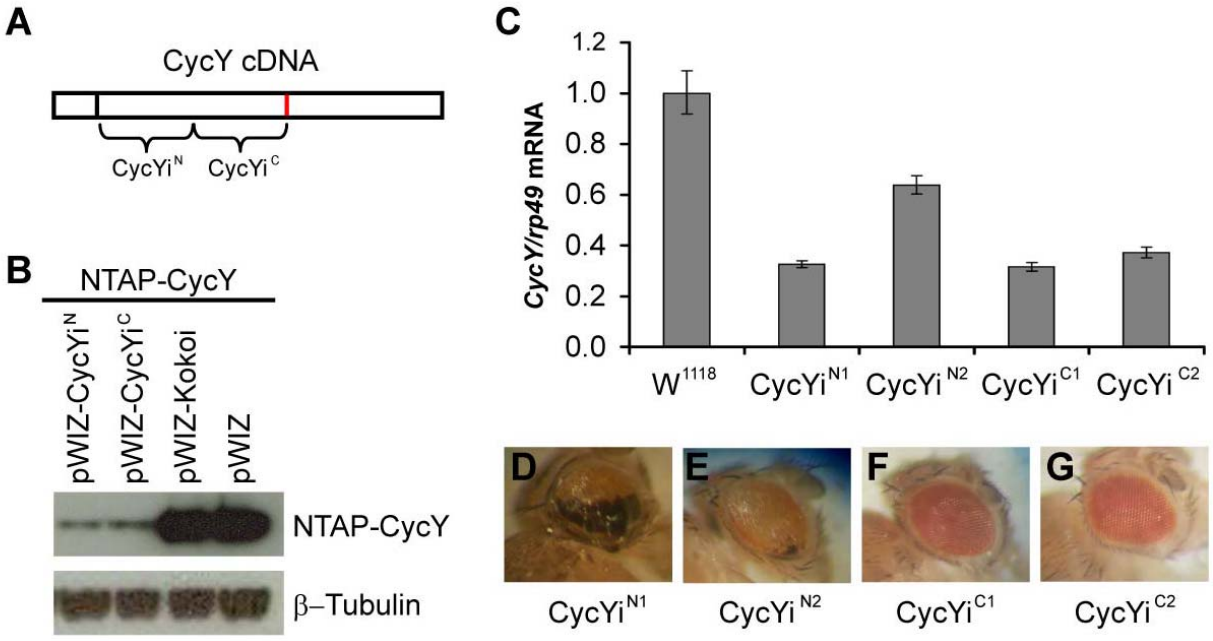


Figure 3-1. Generation of *CycY* conditional knockdown transgenic flies. (A) *CycY* transcript. The cDNA corresponding to the single *CycY* transcript is indicated by the box. The ATG is shown with a back line while the stop codon is indicated with a red line. The coding regions used to generate *CycYi*^{N} and *CycYi*^{C} RNAi constructs are indicated. (B) Western blot to detect the *in vitro* knock down efficiency of exogenously expressed NTAP-CycY by *CycYi*^{N}, *CycYi*^{C}, *Kokoi* (unrelated control), or vector only (pWIZ) in Drosophila S2R+ cells. The immunoblot was probed with antibody to NTAP or β-tubulin. (C) RT-qPCR to detect the *in vivo* knock down efficiency of endogenous *CycY* in second instar larvae by *CycYi*^{N} and *CycYi*^{C} RNAi constructs driven by the ubiquitous driver, Act-Gal4. Two independent insertion lines (N1 and N2 or C1 and C2) were tested for each construct. (D-G) Adult fly eyes harboring the indicated *CycY* RNAi constructs and the *GMR-Gal4* driver.

Expression of *CycYi*^{C} and *CycYi*^{N} in the posterior region of the eye imaginal disc using the *GMR-Gal4* driver (Freeman, 1996) resulted in dramatically different effects. Whereas *CycYi*^{C} did not induce eye defects (Figure 3-1 F-G), *CycYi*^{N} resulted in rough eyes with variable levels of dark pigmentation. Some eyes were yellow to orange with random black spots, while others were totally black (Figure 3-1 D-E and data not shown). The severity of the eye defects induced by different *CycYi*^{N} insertion lines correlated with the level of *CycY* knockdown (Figure 3-1 C-E). The eye phenotype induced by *CycYi*^{N} is unexpected based on the *CycY*^{E8} null mutant, which displayed no eye defects in adult escapers or pharate adults (Liu and Finley Jr, 2010). Thus, I surmise that *CycYi*^{N} may knock down expression of a gene(s) in addition to *CycY*, a possibility that could also explain why ubiquitous *CycYi*^{N} expression leads to earlier lethality than the *CycY*^{E8} mutant. The similarities between the *CycYi*^{C} and *CycY*^{E8} phenotypes on the other hand, suggest that *CycYi*^{C} specifically knocks down *CycY*, which I further confirmed in cDNA rescue experiments described below.

3.3.2 *CycY* is required for wing growth

To test whether *CycY* is required for normal cell proliferation or differentiation I expressed *CycYi*^{C} in cells of the posterior compartment of wing imaginal discs using the *en-Gal4* driver (Brand and Perrimon, 1993; Sigrist and Lehner, 1997). Two independent *CycYi*^{C} insertion lines (*CycYi*^{C1} and *CycYi*^{C2}) were crossed with the *en-Gal4* driver line to knock down expression of *CycY* in the posterior wing. To quantitatively evaluate the effect on wing tissue growth, I measured the size of the wing in the posterior compartment relative to the anterior compartment (P/A). *CycY* knockdown resulted in

smaller posterior wing compartments compared to wild type (Figure 3-2 A-C, I). Two copies of *UAS-CycYi* enhanced the wing size phenotype, as did coexpression of Dicer2, an enzyme that enhances RNAi by cleaving dsRNA into siRNA (Figure 3-2 D-E, I) (Lee et al., 2004). The decreased compartment size could be partially rescued by overexpressing *CycY* with one copy of *UAS-CycY* whereas two copies rescued even better (Figure 3-2 F-G, I), but failed to be rescued by *UAS-GFP* (Figure 3-2 H-I), indicating that the observed phenotype was due to the decreased expression of *CycY* rather than any potential off-target effect.

To determine whether the decreased wing size was the result of decreased cell number, cell size, or both, I measured the relative cell numbers and sizes in defined areas of the anterior and posterior compartments (see Materials and Methods for details). Cell numbers were determined by counting the number of bristles since each wing cell secrets only one bristle during development (Meyer et al., 2000), whereas cell sizes were estimated by the density of cells in a particular area. Decreasing *CycY* expression did not significantly change cell size. The reduced wing size resulting from *CycY* knockdown was primarily due to decreased cell numbers (Figure 3-2J).

To address whether a decrease in cell number caused by loss of *CycY* might be caused by decreased cell proliferation or increased cell death, I also made mitotic clones with the *CycY* deletion mutant, $CycY^{E8}$ (see Chapter 4, section 4.2.10 for details). I stained the wing imaginal discs with $CycY^{E8}$ null clones for phosphorylated histone H3 (PH3) to detect cells undergoing mitosis, or stained the wing imaginal discs with *CycY* knocked down in the posterior compartment by TUNEL approach to detect cells undergoing apoptosis. However, I did not observe any abnormal proportion of mitotic

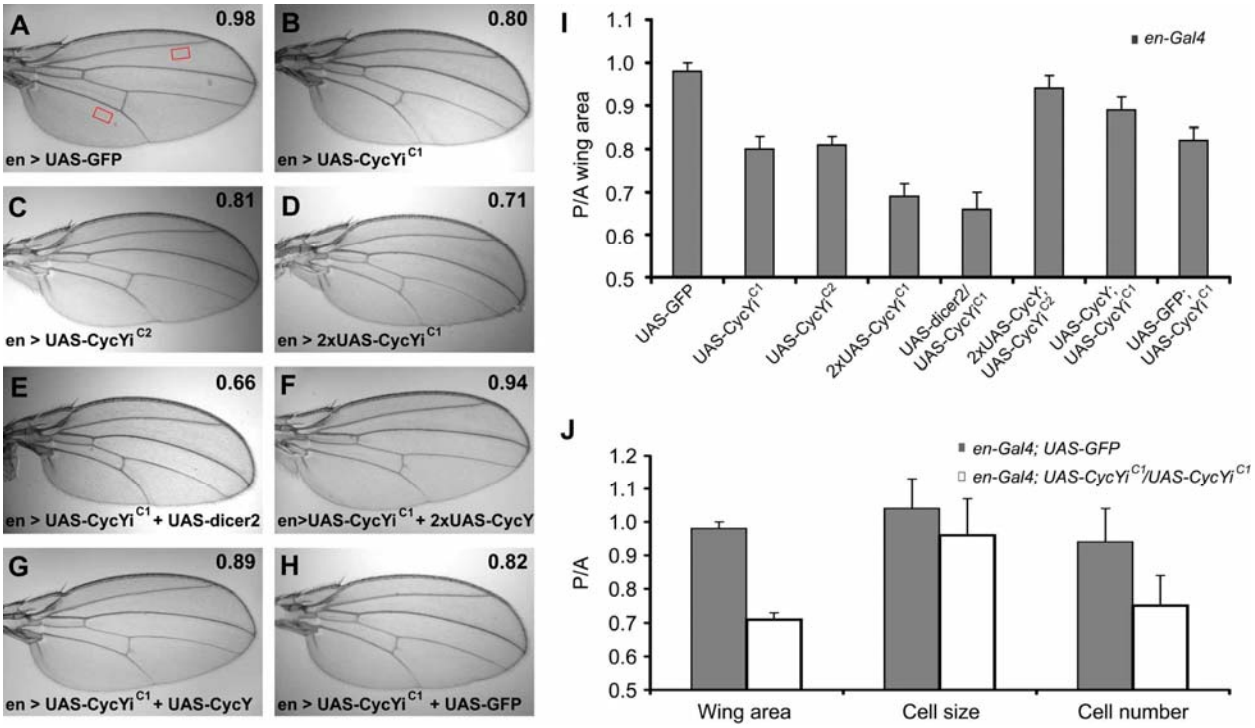


Figure 3-2. CycY is required for wing growth. (A-H) Adult fly wings harboring the en-Gal4 driver and indicated UAS constructs. The number in the upper right corner is the ratio of the posterior to anterior wing sizes (P/A). The red boxes in (A) indicate the defined area for counting the cell numbers in the posterior and anterior region respectively. (I) The P/A area ratio for each indicated genotype. (J) The P/A ratio of wing area, cell size, and cell number for control and *CycY* knock down fly wings.

wing imaginal disc cells in clones lacking *CycY* (Figure 4-5 S-U) or apoptotic wing imaginal disc cells upon knockdown of *CycY* (Appendix J). It is possible that these assays are not sufficiently sensitive to detect changes that lead to only a 20% reduction in cell number.

3.3.3 *CycY* is required for sustained adult viability

I previously showed that *CycY* is required for embryogenesis, larval growth, and metamorphosis, and that it plays a role in oogenesis (Liu and Finley Jr, 2010). To test whether *CycY* is also required during adult life, I rescued $CycY^{E8}$ null mutants into adults by expressing a *CycY* cDNA under control of a heat-shock promoter. Heat shock-induced expression was ceased after eclosion and adult viability was followed for 50 days. $CycY^{E8}$ adults showed reduced lifespan compared with their heterozygous siblings and this effect was more severe for males than for females (Figure 3-3A). This phenotype can be partially rescued by continuously providing CycY in adults by heat-shock treatment of the adults once every day (Figure 3-3B). This finding is consistent with the expression data showing that *CycY* is expressed in both male and female adults and is relatively higher in males (Liu and Finley Jr, 2010). In summary, *CycY* appears to be important throughout the Drosophila life cycle. Using the adult viability phenotype and tissue-specific knockdown should enable exploration of the genetic pathways to which *CycY* may belong.

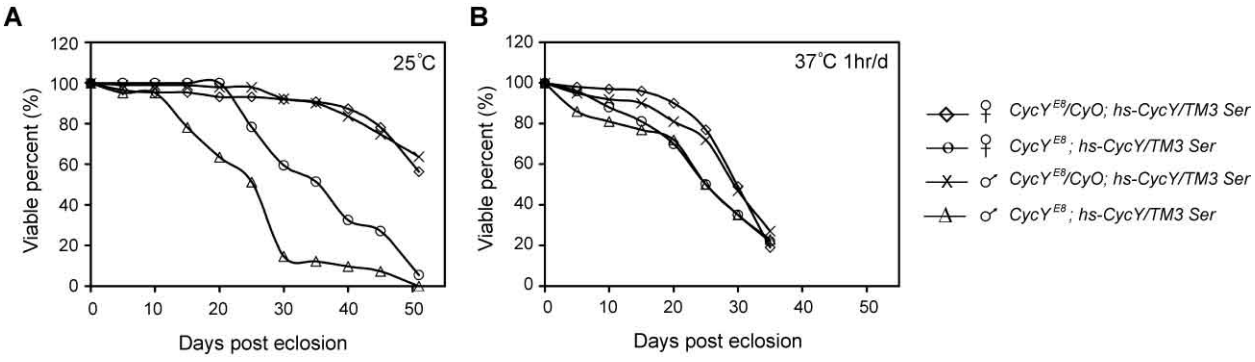


Figure 3-3. *CycY* is required for adult viability. (A) *CycY*^{E8} null mutants that harbor a *CycY* transgene under heat-shock promoter control (hs-CycY) were rescued to adult stage by continuous heat shock treatment once every day after egg laying until adult eclosion to provide exogenously expressed CycY. These *CycY*^{E8} null mutants displayed significantly decreased lifespan relative to their heterozygous siblings. The decrease in life span was more dramatic in males than in females. (B) Continuous heat-shock treatment of adult homozygous *CycY*^{E8} mutants that harbor the hs-*CycY* transgene partially rescued the reduced longevity phenotype. While continuous heat shock reduced viability relative to no heat shock, the difference between homozygous and heterozygous *CycY*^{E8} mutants was minimal.

3.3.4 Snr1 interacts with CycY and is a potential downstream target

To gain insight into the cellular functions of CycY and to identify the signaling pathways to which CycY belongs, I began by examining the available protein interaction data for CycY (Figure 3-4A). Previously, I showed that CycY physically interacts with Eip63E/Cdk14 in Drosophila, which has been supported by studies with human cells (Davidson et al., 2009; Jiang et al., 2009; Liu and Finley Jr, 2010). To focus on potential downstream targets of the CycY/Eip63E complex I excluded physical interactors that are more likely to be upstream regulators. These included other cyclins, the Cdk inhibitor protein Dacapo, and 14-3-3, a scaffold protein that binds to and modulates the function of a variety of signaling proteins, especially their phosphorylated forms (Fu et al., 2000). I searched among the remaining interactors for proteins that may be required at similar developmental times as Eip63E and CycY. Two of the interactors are encoded by novel genes, *CG5783* and *CG8997*, which have not been studied. Another two, PIF-1B, and PIF-2, are cysteine-rich proteins. Both were identified to interact with the non-conserved N-terminal histidine-rich domain of Eip63E. It has been proposed that the function of PIF binding is to counter the inhibitory effect of the long N-terminal extension of Eip63E, which may inhibit the protein's kinase activity (Rascle et al., 2003). Mri is a poorly characterized glycerol kinase. Interestingly, it has been identified in a genome-wide study to be involved in autophagic cell death in salivary glands (Gorski et al., 2003), which is one of the dramatic developmental changes that happen during metamorphosis. The essential requirement of *CycY* and *Eip63E* during metamorphosis makes this interaction a good candidate for future studies. Another Eip63E interactor is Trx-2 (thioredoxin-2), one of the three classical thioredoxins that function to reduce

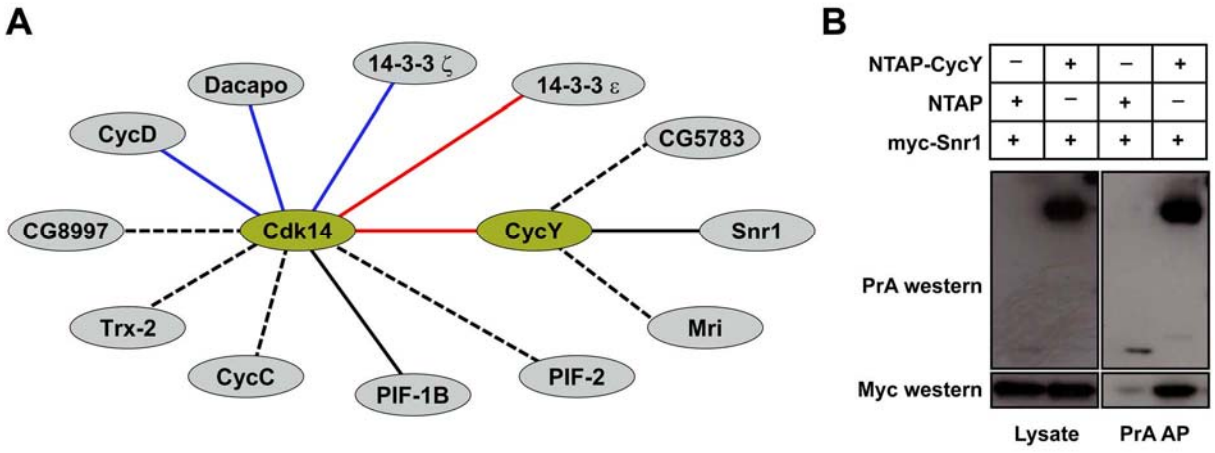


Figure 3-4. CycY physically interacts with Snr1. (A) Protein interactions centered on Cdk14 and CycY in Drosophila. Protein-protein interactions initially detected in yeast two-hybrid screens using Drosophila proteins (black lines), human orthologous proteins (blue lines) or orthologous proteins from both species (red lines) (Davidson et al., 2009; Gao et al., 2006a; Giot et al., 2003; Jiang et al., 2009; Liu and Finley Jr, 2010; Rascle et al., 2003; Shu et al., 2007; Stanyon et al., 2004). The interactions indicated by blue lines are predicted interactions between Drosophila proteins based on the interactions identified with human orthologs. Interactions that have been verified by some other assay such as co-affinity purification are indicated by solid lines. Dashed lines indicate two-hybrid interactions not yet validated by another assay. (B) CycY interacts with Snr1 in Drosophila S2R+ cells. Cells were cotransfected with the indicated constructs and lysed for co-affinity purification (co-AP) using IgG beads.

disulfide bonds in other proteins. Trx-2 has been reported to play a role in adult longevity and tolerance to oxidative stress; homozygous null mutants, however, are viable and fertile (Svensson and Larsson, 2007). The final CycY interactor is Snr1, the Drosophila counterpart of yeast SNF5, a core subunit of SWI/SNF ATP-dependent chromatin-remodeling complex. Snr1 has a number of properties consistent with a possible role in a common pathway with CycY and Cdk14.

First, previous analysis of a temperature-sensitive allele, *Snr1*^{E1}, revealed an essential role for *Snr1* during the window of time one day before and after pupariation (Marenda et al., 2003), which is coincident with the time frame when zygotic *CycY* is critically required (Liu and Finley Jr, 2010). Second, germline clone analysis has shown that *Snr1* is essential for oogenesis (Zraly et al., 2003) and *CycY* is also required for normal oogenesis (Liu and Finley Jr, 2010). Third, *Snr1* mRNA levels oscillate during late larval and pupal development coincident with transient ecdysone pulses (Zraly et al., 2003). Similarly, I have shown that *CycY* mRNA levels oscillate along with ecdysone pulses (Liu and Finley Jr, 2010), while Stowers et al., showed that one of the transcripts of Cdk14/Eip63E is induced by ecdysone (Stowers et al., 2000). Based on the similar developmental requirements and expression profiles for *Cdk14*, *CycY*, and *Snr1*, I hypothesized that these genes belong to a common gene regulatory pathway. As a first step to test this hypothesis, I confirmed the physical interaction *in vitro* by co-affinity purification (co-AP) in Drosophila S2R+ cells (Figure 3-4B). I then screened for genetic interactions between CycY and members of the SWI/SNF complex, which in Drosophila is known as the Brahma (Brm) complex.

3.3.5 CycY genetically interacts with Snr1 and Brm, key members of the Brm complex

It has been reported that Snr1 is required for sustained adult viability (Marenda et al., 2003; Zraly et al., 2003). Dingwall and colleagues constructed and characterized a number of *Snr1* mutants (Dingwall et al., 1995; Marenda et al., 2003; Zraly et al., 2003). One mutant is *Snr1-2*, a putative dominant negative. It was previously shown that overexpression of *Snr1-2* using the *Act5C-Gal4* driver in animals that are heterozygous for a *Snr1* null mutant ($Snr1^{R3}$) dramatically decreases adult viability (Zraly et al., 2003). To test whether *CycY* mutants enhance or suppress the *Snr1* associated viability defect, I combined heterozygous $CycY^{E8}$ null mutants with a combination of the *Snr1-2* dominant-negative and the null allele $Snr1^{R3}$. Ubiquitous expression of *Snr1-2* in the heterozygous $Snr1^{R3}$ background alone at 30°C decreased adult viability significantly in males (Figure 3-5), though this effect was much weaker than previously reported (Zraly et al., 2003). The viability of heterozygous $CycY^{E8}$ mutants was not significantly different from wild-type (Figure 3-5 and data not shown). Strikingly, removal of one copy of *CycY* dramatically enhanced the adult viability defect of the *Snr1* mutants, particularly in males (Figure 3-5).

Another morphological defect induced by ubiquitous expression of the dominant negative *Snr1-2* is incomplete abdominal tergite fusion along the dorsal midline, suggesting functions for Snr1 and presumably the Brm complex in developing histoblasts (Zraly et al., 2003). I used the dorsal midline phenotype as a second assay to test for genetic interaction between *Snr1* and *CycY*. Similar to our observation with the longevity phenotype, when one copy of *CycY* was removed while expressing the

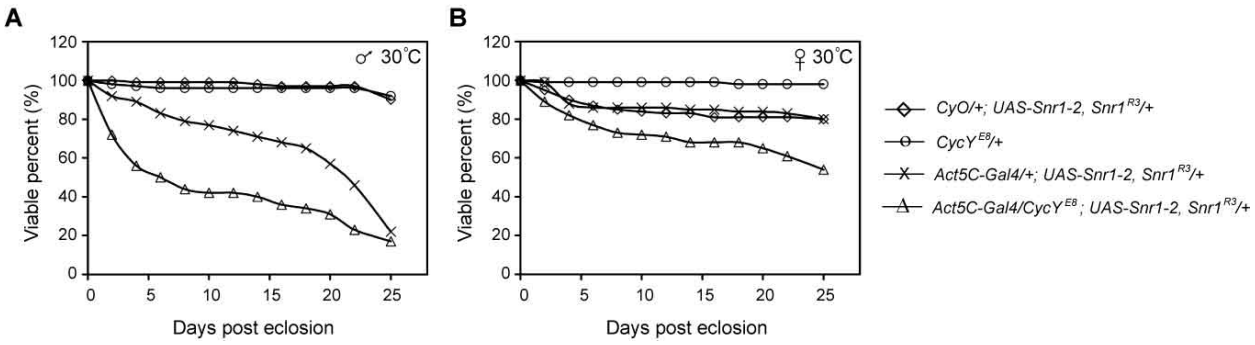


Figure 3-5. *CycY* mutants genetically enhance the reduced lifespan induced by *Snr1* mutants. (A-B) Expression of an *Snr1* dominant negative (*UAS-Snr1-2*) ubiquitously using the *Act5C-Gal4* driver in a heterozygous *Snr1* loss-of-function mutant ($Snr1^{R3}$/+) results in reduced adult viability. Removal of one copy of *CycY* enhanced the adult viability defect in *Snr1* mutants. This effect is more pronounced in males (A) than in females (B).

Snr1-2 dominant negative (in a heterozygous $Snr1^{R3}$ background) I observed a dramatic enhancement of the dorsal midline fusion defect (Figure 3-6, Table 3-2). Since Snr1 is a key subunit of the Brm complex, I wished to ask whether *CycY* also genetically interacts with *Brm*. However, use of the *Act5C-Gal4* driver to ubiquitously knock down expression of *Brm* or *CycY*, or to express the *Brm* dominant negative, Brm^{K804R} resulted in lethality. Thus, it was not possible to test for genetic interactions using the dorsal midline defect and the *Act5C-Gal4* driver. Previously, Zraly et al. showed that expression of *Snr1-2* using the *e22c-Gal4* driver, which is highly expressed in abdomen, also resulted in the dorsal midline fusion defect (Zraly et al., 2003). However, when I used the *e22c-Gal4* driver to express the *Brm* dsRNA, Brm^{K804R}, the $CycYi^{C}$, or to knock down both *CycY* and *Brm* together, I did not observe any dorsal midline fusion defects (Table 3-2). This result suggests that either CycY and Snr1 function independent of the Brm complex in histoblasts, or the *e22c-Gal4* driver is not strong enough to induce any visible phenotype with *CycY* and *Brm* knockdown.

As a further test for genetic interactions between *CycY* and the *Brm* complex I turned to phenotypes generated by knocking down expression in the wing imaginal disc. As discussed above, knocking down *CycY* in the posterior compartment of the wing using the *en-Gal4* driver led to decreased cell numbers and tissue size. I tested whether this phenotype is modified by mutants of *Snr1* or *Brm*. Expression of *Snr1-2* or *Snr1* dsRNA using the *en-Gal4* driver resulted in no change in posterior compartment size (Figure 3-7 A-B) and failed to modify the decreased posterior wing phenotype induced by *CycY* knockdown (Figure 3-7C and D compared with Figure 3-7H and I). Knockdown of *Brm* expression with *en-Gal4* showed a very weak reduction in tissue size (Figure 3-

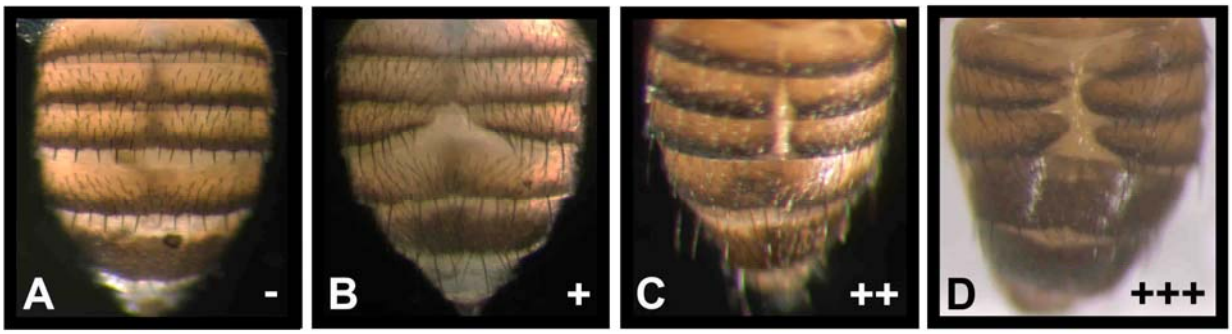


Figure 3-6. *CycY* enhances the dorsal midline fusion defect associated with *Snr1* mutants. (A) Abdominal tergite of a wild type adult. (B-D) Expression of the *Snr1-2* dominant negative in a $Snr1^{R3}$ heterozygous background resulted in incomplete abdominal tergite fusion along the dorsal midline. This phenotype was enhanced by heterozygous $CycY^{E8}$ (Table 3.2). Examples of different levels of severity of the defect are shown; mild + (B), medium ++ (C), and severe +++ (D).

Table 3-2. *CycY* genetically enhances the dorsal midline fusion defects associated with *Snr1* mutants.

Genotype	n	Dorsal midline defects[a]			
		-	+	++	+++
Act-Gal4/CyO; UAS-Snr1-2, $Snr1^{R3}$/TM3 Ser	326	86%	9%	5%	1%
$CycY^{E8}$/CyO	120	100%	0%	0%	0%
$CycY^{E8}$/Act-Gal4; UAS-Snr1-2, $Snr1^{R3}$/TM3 Ser	145	51%	26%	13%	10%
e22c-Gal4/+; UAS-Brm^{K804R}/+	301	100%	0%	0%	0%
e22c-Gal4/+; UAS-Brmi/+	129	100%	0%	0%	0%
e22c-Gal4/+; UAS-$CycYi^{C1}$/+	113	100%	0%	0%	0%
e22c-Gal4/+; UAS-Brmi/UAS-$CycYi^{C1}$	147	100%	0%	0%	0%
$CycY^{E8}$/ e22c-Gal4; UAS-Brmi/+	77	100%	0%	0%	0%

[a] The severity of the dorsal midline fusion defect was quantitatively scored as wild type (-), mild (+), medium (++), and severe (+++). A representative image for each level of severity is shown in Figure 3-6.

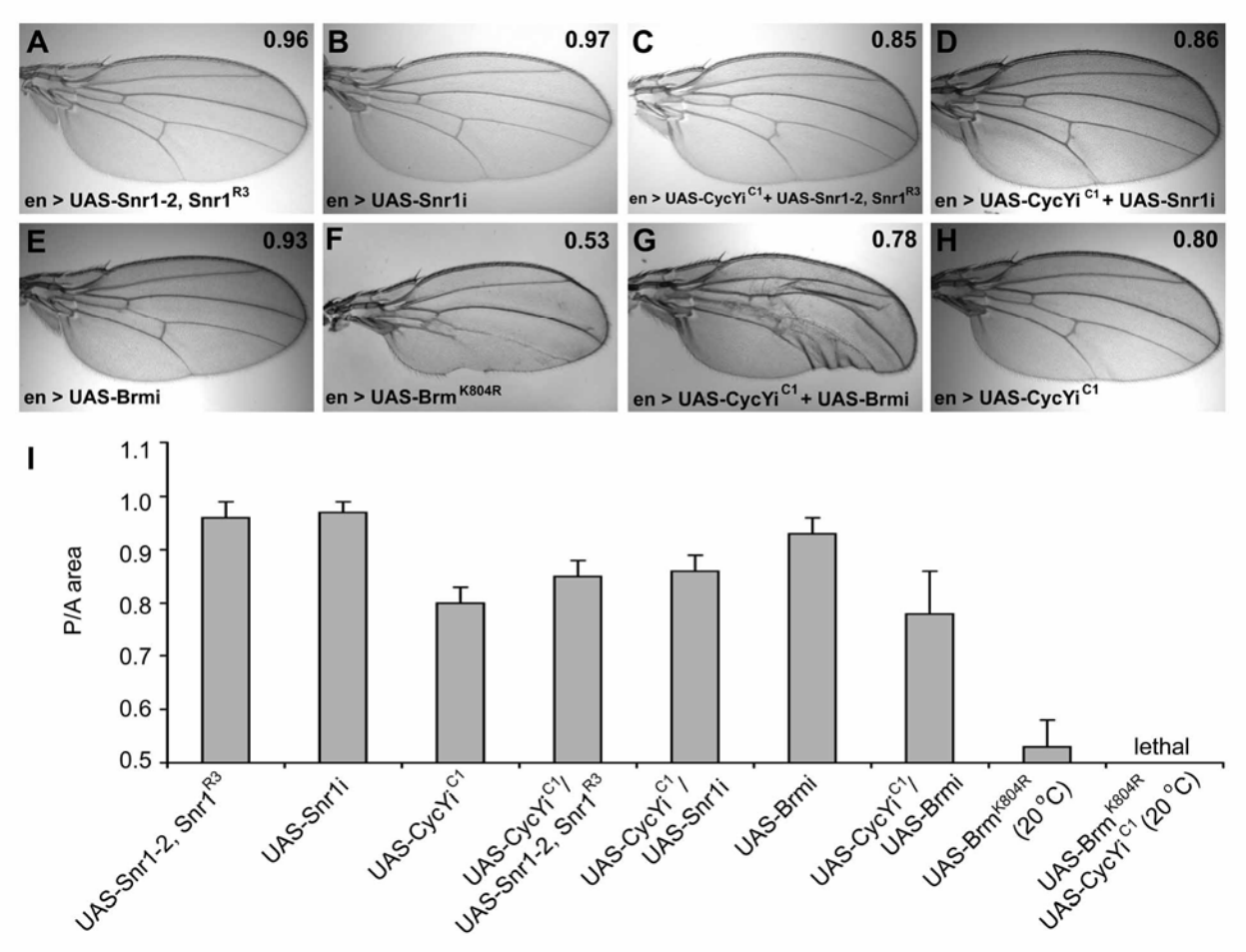


Figure 3-7. Genetic interaction between *CycY* and *Brm* mutants in the wing. (A-H) Adult fly wings harboring en-Gal4 and the indicated UAS constructs. The number in the upper right corner is the ratio of the posterior wing size to that of the anterior (P/A). (I) The P/A area ratio for each indicated genotype. *en-Gal4/+; UAS-CycYi^{C1}/UAS-Brm^{K804R}* are completely pupal lethal.

7E). Knockdown of both *CycY* and *Brm* together also resulted the decreased posterior wing size, but also produced a remarkable novel phenotype. The L4 vein structure was severely disrupted and the overall wing morphology was distorted or crumpled, especially between veins L4 and L5 (Figure 3-7G and I). The loss of vein and crumpled wing blade is similar to the phenotype previously described for knockdown of several Brm complex components, including *Brm*, *Snr1*, *Osa*, and *Mor* using *Sal-Gal4* or *638-Gal4* drivers (Terriente-Felix and de Celis, 2009). The fact that I visualized the same phenotype with a combined knockdown of *Brm* and *CycY* together, but not with knockdown of Brm alone, indicates that loss of *CycY* enhances the Brm knockdown phenotype. I also observed a synthetic lethal genetic interaction between *CycY* and *Brm*. Ectopic expression of Brm^{K804R} by *en-Gal4* was lethal at 25°C. At 20°C, however, I observed some viable adults that showed decreased posterior wing sizes, a notch along the posterior wing margin, and partial loss of L4 and L5 wing veins (Figure 3-7F and I). Knockdown of *CycY* in the same animals resulted in complete lethality. In summary, I observed that knockdown of *CycY* enhances the wing developmental defects induced by both *Brm* knockdown and expression of a *Brm* dominant negative mutant, suggesting that CycY and Brm function cooperatively to regulate some of the same processes in the wing.

To further test for genetic interaction between *CycY* and *Snr1* I used the *69B-Gal4* driver line, which drives expression of *Gal4* throughout most of the wing disc (Brand and Perrimon, 1993). Expression of the *CycY* dsRNA driven by *69B-Gal4* at 25°C induced extra wing-vein tissue to the anterior side of the L2 vein in 10.9% of the animals and extra wing veins on both sides of L2 in a small percentage of the animals

(Figure 3-8 B and C). The extra vein phenotypes can be suppressed by overexpressing *CycY* with a *UAS-CycY* transgene (Table 3-3), indicating that this phenotype is induced by specific knock down of *CycY*. As expected, increasing the temperature to 30°C, which leads to elevated levels of Gal4 (Brand and Perrimon, 1993), resulted in a more severe phenotype than observed at 25°C; for example, after increasing the temperature, the fraction of normal wings went from 88% to 30% while the fraction of wings with extra veins on both sides of L2 went from 0.8% to 29% (Table 3-3). Similarly, knockdown of *Snr1* resulted in an extra wing vein along the anterior side of L2 (Table 3-3), similar to the phenotype previously observed for expression of *Snr1-2* (Zraly et al., 2003) . Knock down of both *CycY* and *Snr1* together significantly enhanced the wing vein phenotype relative to knock down of either gene alone; for example, the fraction of normal wings was reduced from 30 or 50% for *CycY* or *Snr1* knockdown, respectively, to 15% in the double knockdown animals (Table 3-3). Removal of one copy of *CycY*, however, did not modify the *Snr1* phenotype (Table 3-3). Knockdown of *Brm* using *69B-Gal4* was lethal at 30°C, but did not lead to any obvious defects at 25°C (Figure 3-8D). Strikingly, knockdown of *Brm* in combination with *CycY* suppressed the extra wing vein phenotype induced by knockdown of *CycY* and resulted in a highly penetrant novel phenotype. The whole wing blade mildly curved to the ventral side along the midline between the L3 and L4 longitudinal veins. When flattened with a coverslip, a deep crease was evident (Figure 3-8E and Table 3-3). A similar though weaker effect was observed by knocking down *Brm* in a heterozygous $CycY^{E8}$ background (Figure 3-8F and Table 3-3). The fact that *CycY* knockdown and heterozygous $CycY^{E8}$ showed similar but different levels of modification of the *Brm* mutant phenotypes suggests a dosage effect and supports

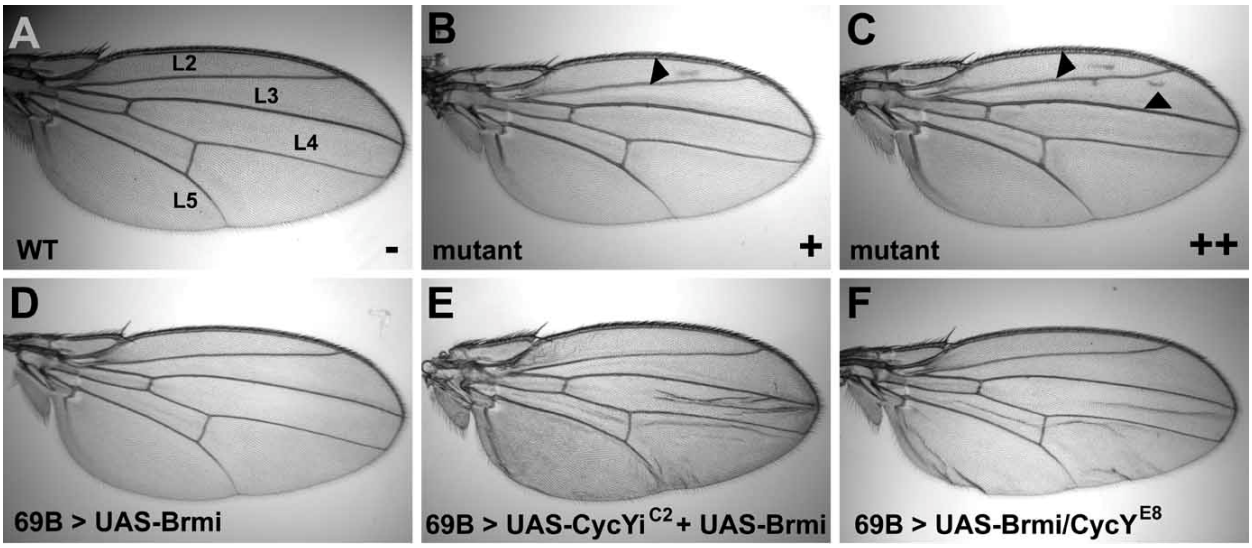


Figure 3-8. Knock down of *CycY* enhances *Snr1* knock down phenotypes and leads to synthetic effects with *Brm* mutants. Knock down of *CycY* or *Snr1* by expressing dsRNA transgenes (*CycYi*^{C} or *Snr1i*) with the *69B-Gal4* driver showed extra wing vein tissue along the anterior or posterior side of L2. Representative wings of wild type "-" (A), extra vein (arrowhead) along one side of L2 "+" (B), and extra vein (arrowhead) along both sides of L2 "++" (C) are shown. (D-F) Wings from *69B-Gal4/UAS-Brmi* (D), *UAS-CycYi*^{C2}*/+; 69B-Gal4/UAS-Brmi* (E), and *CycY*^{E8}*/+; 69B-Gal4/UAS-Brmi* (F) adults reared at 25°C.

Table 3-3. Genetic interactions revealed by double knockdown of *CycY* and *Brm* or *Snr1*.

Temp	Genotype	n	Extra wing vein[a]			Curved wing	
			-	+	++	-	+
30°C	*UAS-CycYi^{C2}/+; 69B-Gal4/+*	237	30.4%	40.9%	28.7%	100%	0%
	69B-Gal4/UAS-Snr1i	795	49.8%	50.2%	0%	100%	0%
	UAS-CycYi^{C2}/+; 69B-Gal4/UAS-Snr1i	127	15%	28.3%	56.7%	100%	0%
	CycY^{E8}/+; 69B-Gal4/UAS-Snr1i	324	52.5%	47.2%	0.3%	100%	0%
25°C	*UAS-CycYi^{C2}/+; 69B-Gal4/+*	222	88.3%	10.9%	0.8%	100%	0%
	UAS-CycYi^{C2}/+; 69B-Gal4/UAS-CycY	172	99.4%	0.6%	0%	100%	0%
	69B-Gal4/UAS-Brmi	114	100%	0%	0%	100%	0%
	UAS-CycYi^{C2}/+; 69B-Gal4/UAS-Brmi	130	100%	0%	0%	0%	100%
	CycY^{E8}/+; 69B-Gal4/UAS-Brmi	147	100%	0%	0%	0%	100%

[a] Wing vein defects were quantitatively scored as wild type (-), extra vein along one side of L2 (+), and extra vein along both sides of L2 (++). A representative image for each class is shown in Figure 3-8 A-C.

conclusion that the *CycY* knockdown effect was specific. The phenotype of double knockdown of *Brm* and *CycY* is similar to the previously described *Contrabithorax* (*Cbx*) phenotype, which is a curving of the wing indicating a partial transformation of wing to haltere due to misregulated *Ubx* expression (Cabrera et al., 1985; Faucheux et al., 2003). Interestingly, Brm^{K804R} mutants exhibit partial haltere-to-wing transformation (Elfring et al., 1998), which is opposite to the curved wing or wing-to-haltere transformation phenotype. Thus, it would seem that Brm knock down and Brm dominant negative mutants display opposite effects with respect to this phenotype. A possible explanation is that the Brm ATPase subunit may have multiple roles in the Brm complex, which can activate some genes but repress others. In the case of Brm knockdown mutants, all function of Brm should be diminished, whereas the dominant negative may only impair a subset of Brm function.

In summary, I detected tissue-specific genetic interactions between CycY and two members of the Brm complex, Snr1 or Brm. Removal of one copy of *CycY* genetically enhanced the defects in adult viability and dorsal midline fusion induced by a *Snr1* dominant negative. Knockdown of *CycY* enhanced the extra wing vein phenotype induced by *Snr1* knockdown and enhanced the wing phenotype of *Brm* knockdown or a *Brm* dominant negative. These genetic interactions indicate that CycY functions with the Brm complex to control specific aspects of wing development.

3.3.6 *CycY* is required for maximal expression of some genes regulated by the Brm complex

The functional relationship between *CycY* and the Brm complex genes may be one where they act in the same pathway to regulate gene transcription. For example, one possibility is that CycY may modulate Brm complex activity to regulate specific genes. To begin to test this possibility, I examined the transcription level of several Brm complex targets in $CycY^{E8}$ homozygous mutants. One set of Brm complex targets appears to be the *Eig71E* genes, a cluster of 11 ecdysone-regulated genes located at cytological position 71E. These genes encode small, secreted, cysteine-rich peptides proposed to be involved in an antimicrobial defense system, based on their sequence similarity to vertebrate defensins (Wright et al., 1996). Both *Snr1* and *Brm* proteins have been detected at the promoters of *Eig71E* genes in Drosophila S2 cells suggesting that these genes are direct targets of the Brm complex. In support of this, Zraly et al. showed that expression of a dominant negative *Brm* in early pupae results in downregulation of most of the *Eig71E* genes, whereas *Snr1* mutants lead to upregulation, further suggesting that Brm and Snr1 have opposing roles in their regulation *Eig71E* genes in pupae (Zraly et al., 2006). To test whether CycY also regulates *Eig71E* gene expression I quantified mRNA levels in early pupae (0-14 hr after puparium formation) that were homozygous or heterozygous for $CycY^{E8}$ (Figure 3-9 A-D). I found that in wild-type pupae, the transcription pattern of *Eig71Eh, Eig71Ei, Eig71Eg, and Eig71Ef* was very similar, with a gradual increase in expression peaking at 6 hr after puparium formation followed by a decrease. In $CycY^{E8}$ mutants, the overall transcription of each gene at most time points was significantly decreased, with the

most dramatic difference evident at the 6 hour peak. This phenotype is similar to *Brm* mutants but opposite to *Snr1* mutants in which these genes are up regulated (Zraly et al., 2006). Interestingly, transcription of three other ecdysone-responsive genes (*Eip93F*, *ImpE2*, *Eip63E*) and the ecdysone receptor gene (*EcR*) were mildly up regulated in the *CycY* mutant (Figure 3-9 E-H). None of these genes has been shown to be markedly affected in either *Snr1* or *Brm* mutants. Combined, these data suggest that *CycY* is required for maximal expression of at least some of the genes that are regulated by the Brm complex.

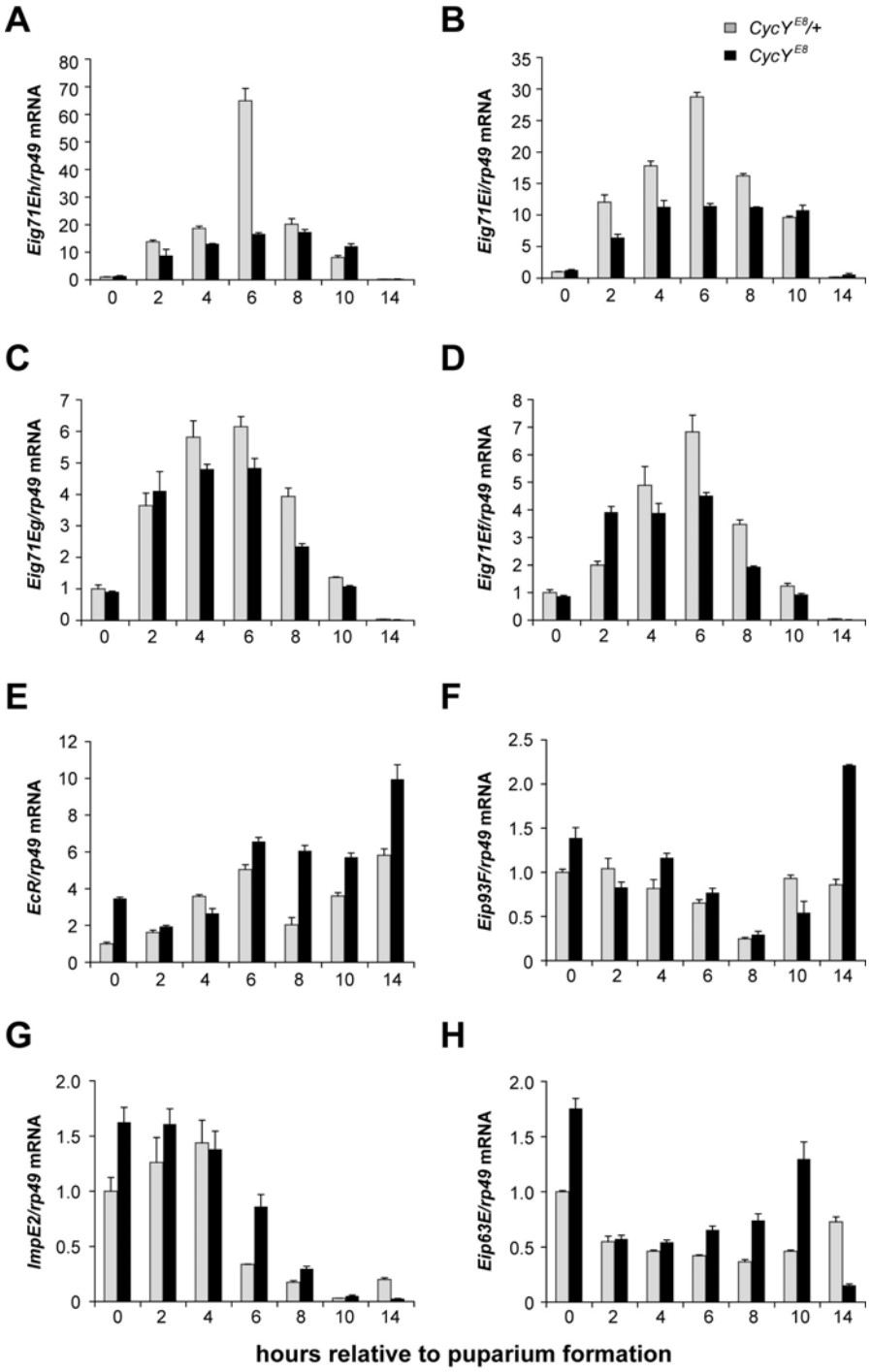


Figure 3-9. Gene expression in *CycY^{E8}* mutants. Total RNA was extracted from prepupae at the indicated developmental time points and mRNA levels of each tested gene were determined by RT-qPCR. Expression was normalized to the mRNA levels of the internal control gene, *rp49*. (A) *Eig71Eh*, (B) *Eig71Ei*, (C) *Eig71Eg*, (D) *Eig71Ef*, (E) *EcR*, (F) *Eip93F*, (G) *ImpE2*, (H) *Eip63E*.

3.4 DISCUSSION

In the current study, I identified additional developmental processes that require *CycY*, including adult viability, abdominal dorsal midline fusion, and wing development. More importantly, I identified that *CycY* genetically interacts with the Brm complex components, *Snr1* and *Brm*, in controlling these developmental processes. The maximum expression of some of the Brm complex target genes, such as *Eig71E* genes, is not achieved in *CycY* null mutants. I also showed that CycY can physically interact with Snr1 in Drosophila cells. Taken together, these results lead us to hypothesize that CycY may modulate the activity of Brm chromatin-remodeling complexes, possibly by phosphorylating Snr1 or other components of the complex, and thereby regulate the transcription of genes involved in a broad spectrum of developmental processes.

3.4.1 CycY may modulate the Brm ATP-dependent chromatin-remodeling complex activity through phosphorylation

CycY and the Brm complex are required for a similar spectrum of developmental processes, consistent with the possibility that they function in a common pathway. Like *Snr1* and *Brm*, *CycY* is an essential gene that is required throughout the Drosophila life cycle, from oogenesis, embryogenesis, and larval growth, to metamorphosis, and adult viability. Mutations in *CycY* and Brm complex components have also revealed common tissue-specific requirements, including development of the integument of the adult abdomen and development of wing veins. The integument of the adult abdomen develops from nests of larval histoblast cells. After a continuous proliferation between 3 and 15 hours after pupariation, the abdominal histoblast cells begin to migrate and

displace the larval epidermal cells, which are then histolyzed by apoptosis (Fristrom and Fristrom, 1993). Dominant negative *Snr1-2* blocked abdominal tergite fusion along the dorsal midline and this phenotype was dramatically enhanced by removal of one copy of *CycY* (Figure 3-6, Table 3-2). A similar abdominal dorsal midline fusion defect has been observed for mutants of the Drosophila caspase, *Ice* (Muro et al., 2006). Thus, it is possible that *CycY* and *Snr1* are involved in histoblast proliferation, migration, or differentiation, or in apoptosis of larval epidermal cells. Clonal analysis with the mutants described in this dissertation may help distinguish these possibilities.

The cell fate determination of wing veins and intervein regions initiates from the larval wing imaginal blade, when broad “provein” and intervein regions are specified by the differential expression of cell signaling molecules, *Hedgehog* (*Hh*) and *decapentaplegic* (*Dpp*), and intervein specific transcription factors, *blistered* (*bs*), *Net*, *Plexus* (*px*), and provein specific transcription factors, *Iroquois* (*Iro*) (for veins L1, L3 and L5), *knirps* (*kni*) and *knirps-like* (*knrl*) (vein L2), *abrupt* (*ab*) (vein L5) (De Celis, 2003). The vein cell fate of each provein is determined by a specific combination of transcription factors. However, vein commitment depends on restriction by *Notch* signaling and activation by *EGFR* signaling pathways. *Snr1* dominant negative mutants display extra wing vein tissue, whereas *Brm* mutants exhibit loss of wing vein, suggesting an inhibitory effect of Snr1 on Brm complex activity in this tissue. Several *EGFR* signaling targets, such as *Delta*, *rhomboid*, and *agros*, and other vein cell fate determination factors or signaling molecules, such as *bs* and *dpp*, were found to be misregulated in mutants of Brm complex subunits (Marenda et al., 2004; Terriente-Felix and de Celis, 2009). Knockdown of *CycY* showed a similar extra wing vein defect to

knockdown of *Snr1. Snr1* knockdown enhanced the extra vein defect induced by *CycY* knockdown (Figure 3-8, Table 3-3). Therefore CycY is also involved in vein cell fate determination, and may function by modulating Brm complex regulation of vein cell fate genes.

Taken together, the similar developmental requirement for CycY and Snr1/Brm, and the genetic interaction between them suggests that CycY may be involved in a common signaling pathway with the Brm complex. However, CycY is unlikely to function downstream of the Brm complex since direct downstream targets of the complex are misregulated in *CycY* mutants (Figure 3-9 A-D). The simplest interpretation of these results is that *CycY* acts in parallel or upstream of the Brm complex. The molecular function of the Brm complex is thought to be primarily transcriptional regulation (Tamkun, 1995) whereas the molecular function of cyclins like CycY is to activate cyclin-dependant kinases to phosphorylate downstream substrates. Our finding that Snr1 and CycY can physically interact (Figure 3-4B) is consistent with the possibility that CycY/Cdk14 phosphorylates either Snr1 or another component of the Brm complex to regulate its activity. Snr1 is phosphorylated at threonine 102 by dDYRK2 and MNB *in vitro* and *in vivo* and this phosphorylation was proposed to regulate the nuclear/cytoplasmic distribution of Snr1 (Kinstrie et al., 2006). In fact, phosphorylation is an important mechanism by which the Brm complex activity is regulated. The human orthologs of Brm (hBrm/Brg1) and Mor (BAF155) have been reported to be inactivated by phosphorylation prior to the onset of mitosis (Muchardt et al., 1996; Sif et al., 1998), a time when chromatin becomes condensed and transcription is inhibited. Brg1 and BAF155 were later shown to be associated with and phosphorylated by CycE/Cdk2

(Shanahan et al., 1999). Thus, it is possible that CycY/Cdk14 also regulates Brm complex activity by phosphorylating one or more of the Brm complex components. In Chapter 4, I describe efforts to detect phosphorylation of Snr1 by CycY/Cdk14.

3.4.2 CycY may modulate Brm complex activity in a cell-type- and temporal-specific manner

Several lines of evidence indicate that the Brm complex can activate or repress transcription of specific genes in a cell-type specific manner. For example, *Eig71E* genes are oppositely regulated in Brm^{K804R} mutant pupae and in *Brm* knockdown Drosophila S2 cells. In Brm^{K804R} mutant pupae, most of the *Eig71E* genes are down regulated, whereas in *Brm* knockdown cells, ecdysone-induced expression of these genes is advanced (Zraly et al., 2006). The expression of another gene, *String* (*Stg*), is also differentially regulated by the Brm complex in different cells. Knockdown of *Snr1* or *Brm* in S2 cells results in down regulation of *Stg* and arrest in G2/M phase (Moshkin et al., 2007). However, in pupae, down regulation of *Stg* was only observed in $Snr1^{E1}$ mutants, but not in Brm^{K804R} mutants (Zraly et al., 2004). More strikingly, the human *Brm* ortholog, *Brg1*, is a well-known tumor suppressor. However, it is also essential for the viability of some types of cells (Mohrmann and Verrijzer, 2005). All of these results might be due to cell type specific activation or repression of specific gene by the ATP-dependent chromatin-remodeling complexes. Snr1, a core subunit of Brm complex, plays the role of an activator or repressor and may be responsible for determining whether the complex activates or represses a gene in a specific cell type. For example, in the intervein cells, *rhomboid* expression is inhibited by Snr1, which blocks the

activation effect of Brm, whereas in the provein cells, *rhomboid* expression is activated by Brm due to the absence of Snr1-recruited repressors (Marenda et al., 2004). Thus, the different regulatory effects of Snr1 may be determined by the availability of tissue-specific transcription factors.

The regulation of Brm complex activity by CycY is also cell-type specific (Figure 3-10). Most of the genetic interaction data indicate that CycY positively regulates Snr1 function, which may either cooperate or antagonize with Brm. For example *CycY* genetically enhances the longevity and dorsal midline fusion defect induced by ectopic expression of truncated *Snr1* (Figure 3-5 and 3-6, Table 3-2). Knockdown of *Snr1* or *CycY* resulted in similar extra wing vein phenotypes, while the double knockdown showed a much stronger effect (Figure 3-8, Table 3-3). CycY appears to activate Snr1 function, whether it is serving to activate or repress genes. For instance, in the intervein cells, Snr1 recruits gene-specific repressors to inhibit the expression of vein-specific genes, and therefore *Snr1* knockdown wings display extra wing vein tissue (Marenda et al., 2004). Knockdown of *Snr1* enhanced the extra wing vein phenotype induced by *CycY* knockdown, whereas *Brm* knockdown suppressed this phenotype (Figure 3-8, Table 3-3). Thus in intervein cells, CycY and Snr1 work together to antagonize Brm. On the other hand, in the provein cells, Snr1 functions with the Brm complex to turn on vein-specific gene expression and this role of Snr1 is also positively regulated by CycY since reducing *CycY* can enhance the loss-of-vein phenotypes induced by *Brm* knockdown (Figure 3-7G). The above model helps explain the tissue-specific genetic interaction between *CycY*, *Snr1*, and *Brm*. Examination of *Eig71E* gene expression in pupae, however, suggests a more complicated relationship. The *Eig71E* genes were

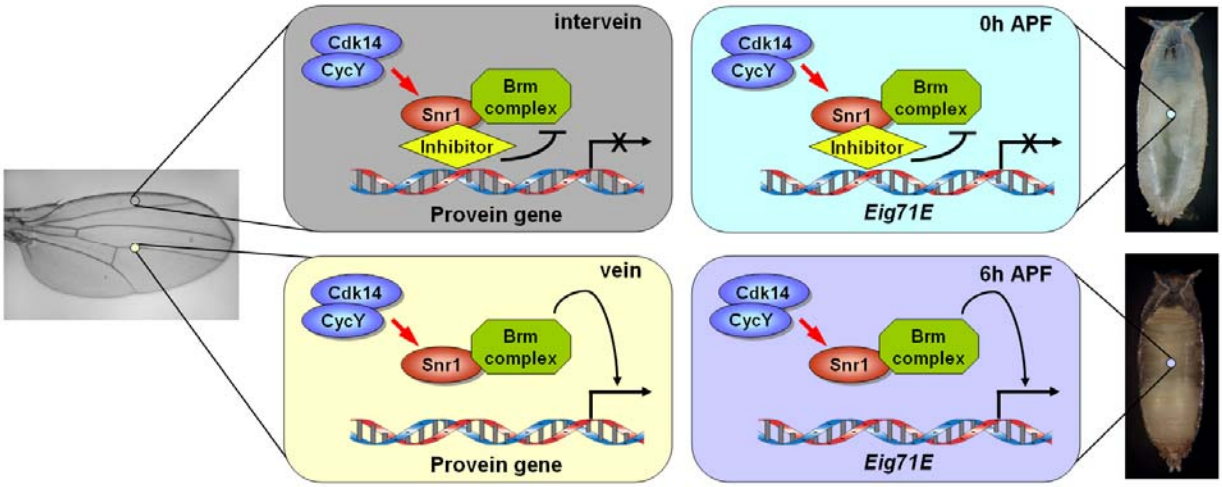


Figure 3-10. CycY may modulate Brm complex activity in a cell-type- and temporal-specific manner. Snr1 may constrain Brm complex activity by recruiting transcriptional inhibitors to repress gene expression at some cell types (for example, intervein cells) or at specific developmental time points (for instance, at 0h after puparium formation or APF) to repress the expression of provein genes or *Eig71E* genes, respectively. Snr1 may also operate with Brm complex to activate gene expression in other cell types (for example, provein cells) or at other developmental time points (for instance, at 6h APF) to activate the expression of these same genes. Under any circumstance, CycY, presumably with its Cdk14 partner, positively regulates Snr1 function to regulate gene expression.

upregulated in the *Snr1*^{E1} mutant, but downregulated in *Brm*^{K804R} mutants, which are a collection of pupae between 0 and 24 hours after puparium formation. My expression data is consistent with previous reports that the expression of *Eig71E* genes is repressed at the beginning of puparium formation, but is induced by ecdysone signaling during the prepupal stage (Wright et al., 1996). Ecdysone-induced expression is downregulated in *CycY*^{E8} mutants, especially at the 6 hour peak. These data seem to conflict with our proposed model that CycY positively regulates Snr1 function. However, it is possible that CycY cooperates with Snr1 to either promote or repress the Brm complex activity in a temporal-specific manner (Figure 3-10). The gene expression analysis with 0-24 pupae may reflect an overall inhibitory effect of Snr1 on Brm complex activity, while my analysis was carried out during the stage of ecdysone-induced expression when presumably Snr1 has an activating role on the Brm complex. Similar analysis of the expression of these *Eig71E* genes at specific developmental time points in *Snr1* or *Brm* mutants is needed to further test this model.

3.4.3 Does CycY play a role in cell cycle regulation?

Thus far it is uncertain whether or not CycY plays a direct role in regulating the cell cycle although I have detected genetic interactions between CycY and the Brm complex, which has been substantially connected with cell cycle regulation. In mammals, the Brm complex cooperates with Rb to inhibit expression of *CycE* and *CycA*, and therefore regulates the exit from G1 and S phase progression. On the other hand, CycE/Cdk2 has been found to bind and phosphorylate Brm subunits Brg1 and BAF155 (orthologs of Drosophila Brm and Mor, respectively) to maintain chromatin in a

transcriptionally permissive state (Reisman et al., 2009). In Drosophila, it has been proposed that the Brm complex may exert an inhibitory effect on DNA replication origins by recruiting a repressor through Snr1. After Cdk2/CycE is also recruited to replication origins by associating with cdc6, Brm complex subunits are phosphorylated by Cdk2/CycE mediated by association with Snr1, and therefore the Brm complex and its inhibitory effect is removed from the replication origins to facilitate the DNA synthesis (Brumby et al., 2002).

Some findings argue against a role for CycY in cell cycle regulation. CycY has not been identified in genome-wide RNAi screens for cell cycle regulators in human or Drosophila cells, and I have found that knockdown of *CycY* expression in cultured Drosophila cells does not produce obvious defects in the cell cycle (data not shown). I did not observe any functions of *CycY* during the development of eye, a best tissue for studying cell proliferation and differentiation. The eyes from $CycY^{E8}$ pharate adults or adult escapers look normal (Liu and Finley Jr, 2010), and knockdown of *CycY* in the posterior compartment of the eye imaginal disc generated viable adults with normal eyes (Figure 3-1 F-G). Cdk14/Eip63E has also been shown not to be required for cell division in the eye (Stowers et al., 2000).

However, several studies support a direct or at least indirect role for CycY/Cdk14 in cell cycle regulation. The decreased wing size phenotype induced by *CycY* knockdown by *en-Gal4* supports a function related with cell growth, cell proliferation, or cell death (Figure 3-2). Unfortunately, I failed to detect any abnormal percentage of mutant cells that undergo mitosis or apoptosis in third instar larval imaginal discs (Figure 4-5 S-U, Appendix J). The human Cdk14 ortholog, also called *PFTK1*, functions

as a Cdk to regulate cell cycle progression, cell proliferation, and activation of Wnt signaling (Davidson et al., 2009; Shu et al., 2007). Two cyclin binding partners for PFTK1 have been identified, CCND3 (human CycD3) and CCNY (human CycY) (Davidson et al., 2009; Jiang et al., 2009; Shu et al., 2007). *PFTK1* knockdown led to G1 arrest, whereas ectopic expression promoted S-phase entry (Shu et al., 2007), suggesting a positive role during the G1 to S phase transition. However, Davidson et al. showed that CCNY oscillates throughout the cell cycle and peaks at G2/M phase (Davidson et al., 2009). Although CCNY/PFTK1 complex peaks at G2/M to activate Wnt signaling by phosphorylating the Wnt co-receptor LRP6, a direct cell cycle regulatory function during G2/M still has not been described. However, direct downstream targets of the Wnt pathway transcription complex β-catenin/LEF-1 include the G1/S genes *CycD1* and *c-myc* (Nollet et al., 1999). It is also not clear whether the cell cycle related PFTK1 kinase activity during G1 to S phase is activated by CCND3, CCNY, or both. It is possible that both CCND3 and CCNY activate PFTK1 but with different substrate specificities. It is possible that CCND3 promotes the phosphorylation of Rb while CCNY promotes the phosphorylation of SWI/SNF complex subunits, and both phosphorylation events would promote the G1 to S phase transition. In Drosophila, however, there is only one CycD, which apparently does not have functions independent of Cdk4, and CycD/Cdk4 complex has been found to be required for cellular growth but not for progression through G1 (Datar et al., 2000; Meyer et al., 2000). Yeast two-hybrid and co-AP data also argue against the interaction between Drosophila CycD and Eip63E (Liu and Finley Jr, 2010). Whether Drosophila CycY and its related kinase are involved in cell cycle regulation in both human and Drosophila requires further investigation.

3.4.4 CycY, Brm complex, and Wg/Wnt signaling

CycY and Cdk14 have been reported to phosphorylate the Wnt co-receptor LRP6/arrow in human and Drosophila cultured cells to activate Wg/Wnt signaling (Davidson et al., 2009). We do not know, however, whether the CycY/Cdk14 complex regulates Wg signaling *in vivo* or whether there are specific developmental processes where CycY/Cdk14 modulate Wg signaling. The genetic interaction between *CycY* and Wg signaling has not been explored. Interestingly, the Brm complex has been found to inhibit the expression of Wg targets (Collins and Treisman, 2000). Thus, it is possible that some or all of the genetic interactions I observed between CycY and the Brm complex are due to CycY's role in Wg signaling. The null mutant phenotypes of *CycY* and *Eip63E* have more similarities to that of Brm complex components than to that of Wg components, suggesting that both CycY and Brm have functions that are independent of Wg signaling. The epistatic relationship between *CycY,* the *Brm* complex, and *Wg* targets should be investigated further. The *CycY* mutants and genetic tools created here should help in this endeavor.

CHAPTER 4

SEARCHING FOR CYCLIN Y'S PATHWAYS IN DROSOPHILA

4.1 INTRODUCTION

The studies described in Chapters 2 and 3 provided an initial characterization of the essential roles that CycY plays in Drosophila and suggested that CycY may bind Cdk14 and activate its kinase activity to regulate the Brm chromatin-remodeling complex. This hypothesis needs to be tested further *in vivo*. The studies also raised many questions about CycY function. In this chapter I set out to answer some of these questions. Most of the results described in this chapter are negative and failed to provide evidence either in support of the hypothesis being tested, or disproving it. Nevertheless the results from these experiments are likely to be useful for guiding future studies aimed at a better understanding of the biological functions of *CycY* and identifying the signaling pathways in which it is involved.

Data presented in Chapter 2 showed that CycY physically interacts with Eip63E as demonstrated by Y2H and co-AP assays. I also showed that *CycY* and *Eip63E* null mutants have similar phenotypes. Both are required for almost all developmental stages. The major developmental defects of zygotic nulls are manifested during metamorphosis, especially during prepupal development, a process that is mainly regulated by the steroid hormone, ecdysone. Interestingly, one of the *Eip63E* transcripts is also ecdysone inducible. Taken together, these data suggest that CycY and Eip63E are *bona fide in vivo* Cyclin/Cdk partners, a proposal that has been further supported by the identification of the CCNY/PFTK1 interaction in human cell lines. However, we still

lack direct *in vivo* evidence from Drosophila. Since I do not have antibodies to either CycY or Eip63E, I was unable to do *in vivo* immunoprecipitation. As an alternative approach, I decided to test whether *CycY* and *Eip63E* genetically interact with each other. The essential requirement of CycY and Eip63E during metamorphosis and the ecdysone induced expression of *Eip63E* prompted me to test whether CycY is required for some key developmental events during metamorphosis, for example the glue protein synthesis, secretion, or extrusion, and autophagic cell death.

Data presented in Chapter 3 showed that *CycY* genetically interacts with components of the Brm chromatin-remodeling complex and that CycY also physically interacts with Snr1, a core subunit of the complex. However, the molecular mechanism behind these interactions is unknown. Considering that the likely molecular function of the CycY/Eip63E complex is to phosphorylate downstream targets, a simple hypothesis is that Snr1 is one of the substrates. To test this hypothesis, I expressed and purified MBP-tagged Snr1 and several mutant variants and used these fusion proteins as substrates for *in vitro* kinase assays.

I show here that *CycY* does not genetically modify the hatching rate defect associated with $Eip63E^{81}$ allele. I also show that *CycY* null mutants display normal starvation induced autophagy during early L3 stage. Interestingly, the process of glue extrusion, which is regulated by ecdysone, is misregulated in the *CycY* mutant. Due to the failure of generating high quality CycY Ab and the non-specificity of RNA *in situ* hybridization, I was unable to show the endogenous *CycY* protein or mRNA localization. Consistent with studies with human cells, I showed that CycY with an N-terminal tag is mainly localized in the cytoplasm. Finally, *in vitro* kinase assays suggest that Snr1 may

not be a direct phosphorylation target of CycY/Eip63E complex. This result suggests an alternative hypothesis for how CycY/Cdk14 may regulate the Brm complex.

4.2 MATERIALS AND METHODS

4.2.1 Fly stocks

All fly stocks were maintained in vials containing standard cornmeal molasses medium and raised at 25°C unless otherwise stated. Most of the fly strains used in this study have larval markers for identifying larvae with specific genotypes. A double balancer strain that have a [*y*+] transgene on the second chromosome in a y^1 background and a *Tb* allele on the third chromosome was kindly provided by Dr. Lei Zhang (University of Texas Southwestern Medical Center; Shanghai Institute of Biochemistry and Cell Biology) (Finley lab # 442). Fly strains used in this study are listed in Appendix K.

4.2.2 Embryo hatching rate

Roughly equal numbers of newly emerged virgin females (~20) of each genotype were mated with w^{1118} males (~30) or males with the indicated genotypes in a regular vial for 2 days. These adults were then transferred to egg chambers after 2 days of mating and eggs were collected on apple juice plates once every 12 hours for two days. After incubating the collected eggs for about another 12 hours, the number of hatched first instar larvae was counted. The hatched larvae were discarded after counting. The number of hatched first instar larvae was counted again after another 12 hours. The reason for counting twice is to prevent too many larvae from accumulating on the apple juice plates since they will crawl to the cover of the plate and dry out or dig through the agar when it is over crowded. The number of eggs that failed to hatch eventually after

aging for at least 24 hours at 25°C was also counted. Hatching rate is the total number of hatched first instar larvae divided by the sum of larvae and unhatched eggs.

4.2.3 Assay of glue synthesis and extrusion from salivary glands during prepupal development

Egg chambers were set up with Finley lab strain # 710 (*y^1w^*; $CycY^{E8}$/CyO* [*y+*]*; sgs3-GFP/TM6b Tb*). Larvae with black mouth hooks (*y^1w^*; $CycY^{E8}$/CyO* [*y+*]*; sgs3-GFP/TM6b Tb or y^1w^*; $CycY^{E8}$/CyO* [*y+*]*; sgs3-GFP)* and yellow mouth hooks (*y^1w^*; $CycY^{E8}$; sgs3-GFP/TM6b Tb or y^1w^*; $CycY^{E8}$; sgs3-GFP)* were collected separately and transferred to regular vials. 0h prepupae were collected and transferred to a petri dish with a piece of wet filter paper. The glue secretion from salivary glands was monitored under a dissection fluorescence microscope once every half hour for a total of 4 hours. All the pupae under study were classified into five different groups on the basis of the GFP intensity, which represents the glue protein sgs3 level, during the first four hours after puparium formation. Group 1 had lost almost all GFP; group 2, had only a weak GFP signal; group 3 had medium levels of GFP signal; group 4 had strong GFP signal at the time of puparium formation, but the signal went away in less than 1.5h; group 5 had GFP signal that remained longer than all the above mentioned situations.

4.2.4 LysoTracker staining

An egg chamber was set up with Finley lab fly strain # 693 (*w^*; $CycY^{E8}$/CyO Act5C-GFP*). *$CycY^{E8}$* heterozygous (GFP positive) and homozygous (GFP negative) first instar larvae were transferred separately into regular vials (about 20 per vial) from apple

juice plates. Well-fed early third instar larvae were collected for direct dissection or were transferred into 5ml of 20% sucrose/PBS for 4 hours before dissection for protein starvation experiments (Britton and Edgar, 1998). I followed the LysoTracker staining protocol as described by Neufeld with minor modification (Neufeld, 2008). Early third instar larvae were dissected in a 6-well dissection dish containing PBS and the dissected salivary glands and fat body tissue that were attached to the mouth hooks were immediately transferred to an eppendorf tube with PBS on ice. After finishing the dissection of all larvae (~10), PBS was removed and replaced with 1ml of 2 μg/ml Hoechst 33342 (Invitrogen H1399, 1mg/ml stock solution). After 10-minute incubation, Hoechst 33342 was removed and the dissected tissues were washed twice with PBS (5 minutes for each wash). The eppendorf tube was kept on the bench without shaking during the washing step. After washing with PBS, the tissues were incubated with 1ml of 1μM LysoTracker Red DND-99 (Molecular Probes L-7528, 1mM stock solution) for 2 minutes. After washing twice with PBS (5 minutes for each wash), the salivary glands and fat body were mounted with PBS and photographed immediately.

4.2.5 Expression and purification of MBP-tagged fusion proteins from bacterial cells

I followed the following protocol, which I modified based on the NEB instruction manual for pMAL Protein Fusion and Purification System. Transform the expression vector into NEB express cells (*NEB C25231, fhuA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10--TetS)2 [dcm] R(zgb-210::Tn10--TetS) endA1 Δ(mcrC-mrr)114::IS10*). Inoculate 10ml of rich media (per liter: 10g tryptone, 5g yeast extract, 5g NaCl, 2g

glucose, 100μg/ml ampicillin) with a single colony. Inoculate 1L of rich media with the 10ml of overnight culture and grow to OD_{600} ~0.6, which usually takes about 2.5-3h. Add IPTG to a final concentration of 0.6mM to induce the fusion protein expression and incubate with shaking at 37°C for 4 hours. Spin down and measure the weight of the wet pellet. Resuspend 1g pellet in 10 ml of lysis buffer [1xPBS, 1mM PMSF, 1mMEDTA, 1xProtease inhibitor cocktail (Roche)]. Add rLysozyme (Novagen) ~ 50KU/g cell pellet and shake at room temperature for 1h (200RPM). Store the cell lysate at -20°C freezer over night. Thaw rapidly at 37°C in a water bath and then return to ice. Transfer the cell lysate to a 50ml centrifugation tube and keep the volume around 20ml, no more than 25ml. Sonicate on ice (8 x 15 seconds each, pulsed 15s in between, duty cycle 50%, setting 5, microtip max). Spin at 17,000 rpm at 4°C for 30 min. Filter the supernatant with a 0.4μm membrane. Quantify protein concentration with the BioRad protein assay reagent. Dilute the supernatant to 2.5mg protein/ml with MOPS column buffer (50mM MOPS pH 7.5, 200mM NaCl, 1mM EDTA, 1mM PMSF). Load to a 15ml amylose (NEB) column (1ml/min) equilibrated with 150ml of MOPS column buffer. Wash the column with 180ml of MOPS column buffer (3ml/min). Elute MBP-tagged fusion proteins with the MOPS column buffer supplemented with 10mM maltose. Collect 15 fractions (1ml/min), each ~ 1.5ml. Quantify the protein concentration in each fraction and run an SDS-PAGE gel and stain with Coomassie to identify the purified protein fractions. The following MBP-fusion proteins have been expressed and purified for this study (Table 4-1).

Table 4-1. MBP-fusion proteins expressed and purified for this study

Plasmids Finely lab #	Plasmid names	Expressed proteins	Used for
1030	pMalc2-nCycY	MBP-nCycY	CycY Ab purification
1010	pMalc2-Snr1	MBP-Snr1	Substrate for kinase assay
1033	pMalc2-Snr1 T102/198A	MBP-Snr1 T102/198A	Substrate for kinase assay
1034	pMalc2-Snr1 T102A	MBP-Snr1 T102A	Substrate for kinase assay
1035	pMalc2-Snr1 T198A	MBP-Snr1 T198A	Substrate for kinase assay

4.2.6 Generation and purification of CycY peptide antibody

A 14 amino acid small peptide DRKSKDMPPVFEER (CycY 16-29) was synthesized and used for the immunization of a guinea pig by Open Biosystems. Forty five ml of crude serum was received and directly used for western blot (1:250) to detect CycY. To purify the anti-CycY serum, a small MBP-tagged N-terminal CycY (1-115, MBP-nCycY; expression vector pMalc2-nCycY is Finley lab plasmid # 1030) was expressed in *E.coli*, purified (see section 4.2.5 for details), and coupled to the Affi-Gel 15 (Bio-Rad). Affinity purification of CycY antiserum is based on the manufacturer's protocol (Bio-Rad).

4.2.7 RNA *in situ* hybridization

I followed the following protocol adapted from the protocol provided by Dr. Ye Tao (The University of Texas M. D. Anderson Cancer Center) and Dr. Russell Finley (WSU).

4.2.7.1 Collect and fix embryos for hybridization

Set up an egg chamber and collect eggs at room temperature (22°C) for 20-24 hours. Wash eggs off the egg laying plate into a sieve and wash several more times to remove as much debris as possible. Immerse the eggs in the sieve in 50% bleach for 5 minutes to dissolve the egg chorions. Wash several times with water and blot dry. Using a paint brush with soft hair, transfer the eggs immediately into a glass scintillation vial containing equal volumes of fixative solution (3.7% formaldehyde in PEM; PEM: 100mM PIPES, 1mM $MgCl_2$, 1mM EGTA, pH 6.9) and heptane; place the glass vial on a shaker and shake at 400 rpm for 20 minutes at room temperature. Using a pasteur pipet,

remove and discard the lower phase and add an equivalent volume of methanol (precooled to -70°C) into the vial. Shake the vial vigorously by hand for 1 minute. Discard the upper phase in the vial, and any embryos that remain at the interface should be discarded as well. Remove the embryos from the bottom phase and transfer into a 1ml tube. Rehydrate the embryos through a graded methanol:PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, pH7.4) series (7:3, 5:5, 3:7, 0:10), 10 minutes each. Fix the embryos for 20 minutes with 3.7% formaldehyde. Wash in 1xPBS, then dehydrate through graded methanol:PBS series (3:7, 5:5, 7:3, 10:0), 10 minutes each. Store the embryos at -20°C for further treatment.

4.2.7.2 Probe synthesis

Using pAS1-CycY as template, PCR with primers 5' TGTGGGATGACCAGGCC GTTTGG (Finley lab # 792) and 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGG AGGCGGCGGAATTAGCTTGGCTGCAG-3' (Finley lab # 497) to amplify a *CycY* cDNA fragment (C-terminal 359bp) with a T7 promoter at the 3’ end. Alternatively, PCR with primers Finlab # 497 and 5’RT-F (Finley lab # 891) to amplify full length *CycY* cDNA with a T7 promoter at the 3’ end. Set up the following RNA labeling reaction with the DIG RNA Labeling Kit (SP6/T7) from Roche Applied Science (11-175-025-910): 10µl of PCR products from above (~ 1µg DNA); 2µl of 10 x Transcription buffer; 2µl of l0x nucleotide mix (with digoxigenin-UTP); 1µl of RNase inhibitor; 2µl of T7 RNA polymerase. Add RNase-free water to give a final reaction volume of 20µl. Incubate the reaction mixture at 37°C for 2 hours. Clean up the *in vitro* synthesized labeled RNA probes with Megaclear RNA purification kit (Ambion). Add 2µl of RNase inhibitor (40U) to the purified RNA probe. Aliquot the probe and store at -70°C.

4.2.7.3 Preabsorb anti-Dig Ab

Remove methanol from the fixed embryos (~50µl). Add 1ml of PTW (PBS, 0.1% Tween-20) and 5µl of Roche HRP conjugated anti-digoxigenin antibody. Rotate at 4°C overnight. Add NaN_3 to the preabsorbed Ab solution to a final concentration of 0.1% to stabilize the Ab. Preabsorbed Ab may be stored for several months at 4°C and diluted further 1:10 when used.

4.2.7.4 Pretreatment, hybridization and washing

Rehydrate the fixed embryos through 70%, 50%, 30%, 0% methanol in PTW (PBS, 0.1% Tween-20). Five minutes for each step. Incubate the embryos in 50µg/ml proteinase K in PTW at room temperature for 4 minutes. Rinse the embryos twice for 1 minute in PTW. Wash with PTW twice for 5 minutes. Fix the embryos in 4% paraformaldehyde in PBS for 20 minutes at room temperature. Rinse the embryos five times for 1 minute in PTW.

Rinse the embryos in 1:1 PTW: hybridization buffer (50% deionized formamide, 5x SSC, 100µg/ml tRNA, 50µg/ml heparin, 0.1% Tween-20; SSC: 0.15M NaCl and 15mM tri-sodium citrate; adjust pH to 4.5 with 1M citric acid if the hybridization temperature is 70°C, otherwise do not adjust pH). Resuspend the embryos in hybridization buffer and transfer to a 0.6ml tube. Prehybridize overnight (~20h) at 70°C or 55°C. Heat the probe (1:25-1:100 in hybridization buffer) to 90°C for 5 minutes. Put the heated probe on ice immediately. Remove prehybridization buffer from the 0.6ml tube and add 200µl fresh hybridization buffer containing heat denatured probe. Hybridize overnight in 70°C or 55°C water bath.

Remove hybridization buffer containing the probe and rinse the embryos in fresh hybridization buffer once and wash once for 20 minutes at 70°C or 55°C. Wash the embryos in 1:1 PTW: hybridization buffer for 20 minutes at 70°C or 55°C. Wash in PTW five times for 20 minutes at room temperature.

4.2.7.5 Immunodetection of the probes

Incubate the hybridized embryos with preabsorbed anti-dig Ab (further dilute 1:10 in PTW to make a final 1:2000) for 2 hours at room temperature. Wash four times for 20 minutes in PTW. Rinse twice for 1 minute in fresh-made detection solution (100mM NaCl, 50mM $MgCl_2$, 100mM Tris-HCl pH 9.5, 0.1% Tween-20). Resuspend the embryos in 1ml of detection solution and add 20µl of NBT/BCIP mixture solution (Roche). Monitor the reaction under the microscope. When the stain appears, stop the reaction with several rinses in PTW. Mount the embryos in 70% glycerol. Use small pieces of No.1 coverslip as supports for the main coverslip to prevent the embryos from being squashed.

4.2.8 Immunofluorescence staining of Drosophila S2R+ cells

I followed the following protocol provided by Bridget Elsa Varughese (WSU).

4.2.8.1 Coat coverslip with Concanavalin A (ConA)

Immerse coverslips into nitric acid for about 2 minutes. Wash with abundant amounts of sterile water. Make sure the final pH of the wash is ~neutral. Dry the coverslips on paper towel and put one in each well of a 6-well plate. Spread 100-200µl ConA (250µg/ml, Sigma C5275) on each coverslip. Incubate at 37°C for 2 hours.

Remove the extra ConA with a pipette and rinse with sterile dH_2O 3 times. Store the coverslips in water at 4°C for future use.

4.2.8.2 Seed cells

Dislodge S2R+ cells and count the cell density. Dilute the cell suspension to $5x10^5$ cells/ml with fresh media. Remove extra water from the 6-well plate with ConA-coated coverslips. Plate 200µl of the cells (10^5 cells in total) onto each coverslip. Wait 30min for the cells to settle down. Remove excess cell suspension form the coverslip with a pipette.

4.2.8.3 Staining

Fix the cells with 1ml of 4% paraformaldehyde (pre-warmed at 37°C) for 10 min at room temperature. Wash 3 times with 1xPBS (10 minutes for each wash). Block with 1ml of 0.1% PBT (Triton), 5% BSA for 1h at room temperature or 4°C overnight or longer. Add 200µl of 1°Ab (mouse anti-myc Ab 1:100, Santa Cruz; rabbit anti-GST Ab 1:100, Santa Cruz) to a piece of parafilm then put the coverslip on the top of it with the cells facing down. Incubate at 4°C overnight or for 1-3h at room temperature. Wash 3 times with 1xPBS (10 minutes for each wash). Add 200µl of 2°Ab (FITC conjugate Goat anti-mouse IgG 1:200, Sigma; Texas Red® goat anti-rabbit IgG 1:200, Invitrogen) to a piece of parafilm then put the coverslip on the top of it with the cells facing down. Incubate at room temperature for 1h. Wash 3 times with 1xPBS (10 minutes for each wash). Mount with vectashield mounting media with DAPI. Seal the edge with nail polish. Keep slides at room temperature in the dark overnight to fully dry. Stained slides can be stored at 4°C in the dark for months. Images were taken with a Zeiss Axio imager upright microscope and Zeiss AxioCam MRm camera.

4.2.9 Immunoprecipitation and kinase assay

I followed the following protocol provided by Dr. Stephen Guest (WSU) with minor modification. Transfect S2R+ cells with plasmids and prepare cell lysate as described in Chapter 2 (section 2.2.11). Quantify protein concentration in the cell lysate and aliquot 500μg of total protein to an epppendorf tube. Add fresh-made lysis buffer (20mM Tris.HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1mM NaF, 1mM Na_3VO_4, 1mM β-Glycerophosphate, 1 x protease inhibitor cocktail, 1mM PMSF) to make it a total of 1ml. Add 10μl of anti-myc Ab (Santa Cruz) to the above cell lysate and rotate at 4°C for an hour. Add 20μl of protein A agarose beads (Santa Cruz sc-2001) and continue to incubate on a nutator at 4°C overnight. Centrifuge immunoprecipitation reactions at 2500rpm at 4°C for 5 minutes. Remove supernatant and wash the beads 3 times with kinase assay buffer (50mM Hepes pH7.5, 10mM $MgCl_2$, 1mM DTT). After the final wash, add 20μl of kinase reaction mix to each immunoprecipitate (1 x kinase assay buffer, 50μM cold ATP, 4.5μCi [γ^{32}P] ATP, and 2μg of potential kinase substrate). Incubate at 30°C for 30 minutes. Stop the reaction by adding 20μl of 2 x Laemmli sample buffer and analyze by SDS-PAGE.

4.2.10 Mosaic analysis

y^1w^* hs-FLP; Ubi-GFP FRT40A (Finley lab # 434) virgin females were crossed with *y^1w^**; *$CycY^{E8}$ FRT40A/CyO* [*y+*]*; TM6B Tb/Sb* (Finley lab # 705) males or *y^1w^**; *FRT40A/CyO* [*y+*]*; TM6B Tb/Sb* (Finley lab # 704) males as control in glass vials. Eggs were collected for 24 hours. Twenty-four and 48 hours after egg laying, the glass vials

were heat-shocked in a 37°C water bath for 2 hours to initiate FRT-mediated mitotic recombination (Xu and Rubin, 1993). These vials were kept at 25°C otherwise. Third instar larvae with yellow mouth hooks (*y^1w^* hs-FLP; CycY^{E8} FRT40A/Ubi-GFP FRT40A or y^1w^* hs-FLP; FRT40A/Ubi-GFP FRT40A*) were collected and dissected. Dissected imaginal discs were fixed in 4% paraformaldehyde in PBS for 30 minutes at room temperature. After washing 3 times with PBST, staining 10 minutes in 1μg/ml DAPI/PBST (Sigma), and a final 10-minute wash in PBST, imaginal discs were incubated with antifade solution (0.5% p-Phenylenediamine and 40% glycerol in PBS) for 20 minutes and mounted on a slide. Similarly, *y^1w^* ey-FLP; Ubi-GFP FRT40A* (Finley lab # 435) virgin females were crossed with *y^1w^**; *CycY^{E8} FRT40A/CyO* [*y*+]*; TM6B Tb/Sb* (Finley lab # 705) or *y^1w^**; *FRT40A/CyO* [*y*+]*; TM6B Tb/Sb* (Finley lab # 704) males to create mosaic clones specifically in the eye.

4.3 RESULTS AND DISCUSSION

4.3.1 *CycY* does not genetically modify the hatching rate defect associated with the *Eip63E*^{81} allele

I have shown that CycY physically interacts with Eip63E and that null mutants of the two genes have strikingly similar phenotypes, suggesting that CycY and Eip63E function together *in vivo*. To further test this possibility I set out to screen for a genetic interaction between the two genes. Since there is a maternal requirement for both *CycY* and *Eip63E* during embryogenesis, I compared the hatching rate of embryos from female heterozygous for *Eip63E* or *CycY* mutants with that of double mutants when mated with *w*^{1118} males (Table 4-2). However, I only identified a decreased hatching rate associated with the *Eip63E*^{81} allele, but not with any allele of *CycY* or the other two *Eip63E* deficiency alleles. Embryos from double heterozygous mutants (*CycY*^{E8}/+; *Eip63E*^{81}/+) had similar hatching rate as those from the heterozygous *Eip63E*^{81} mutant. The same was true when the father was heterozygous for a *CycY* allele, an *Eip63E* allele, or both. These data suggested that the decreased hatching rate is an allele-specific phenotype of *Eip63E*^{81} that is not modified by decreasing the dosage of *CycY*. There are several possibilities to explain the differences between the *Eip63E*^{81} allele and the two *Eip63E* deficiency lines. *Eip63E*^{81} has an in frame deletion of the DNA sequences that encode amino acids 226-241 within a region conserved among Cdks, including the highly conserved lysine 234, which is essential for ATP binding. Stowers et al. proposed that *Eip63E*^{81} is a null (Stowers et al., 2000). However, it is more likely to be a dominant negative since this truncated Eip63E may maintain its ability to interact

Table 4-2. Embryo hatching rates of *CycY* and *Eip63E* mutants.

Maternal genotype	Paternal genotype	Embryos collected	Hatching rate (%)
$CycY^{E8}$/+	w^{1118}	906	98
$CycY^{Df6030}$/+	w^{1118}	721	99
$Eip63E^{81}$/+	w^{1118}	2273	73
$Eip63E^{Df6096}$/+	w^{1118}	771	98
$CycY^{E8}$/+; $Eip63E^{81}$/+	w^{1118}	2519	74
$CycY^{Df6030}$/+; $Eip63E^{Df6096}$/+	w^{1118}	1184	98
$CycY^{Df6030}$/+; $Eip63E^{Df6095}$/+	w^{1118}	1030	99
$CycY^{Df6030}$/+	$CycY^{E8}$/+	772	98
$Eip63E^{81}$/+	$Eip63E^{Df6096}$/+	1178	75
$CycY^{E8}$/+; $Eip63E^{81}$/+	$CycY^{Df6030}$/+; $Eip63E^{Df6095}$/+	3127	78
$CycY^{E8}$/+; $Eip63E^{81}$/+	$CycY^{Df6030}$/+; $Eip63E^{Df6096}$/+	1888	78

with the cyclin binding partner, even though it has lost its kinase activity. This may cause the different behaviors of *Eip63E*^{81} and the deficiency. It is also possible that there is a second site mutation on the *Eip63E*^{81} chromosome that contributes to or causes the hatching defect. Although these results did not provide further evidence to support the idea that CycY and Eip63E function together, the failure to detect a genetic interaction does not weaken the hypothesis. For interaction partners, lowering the dosage of the two proteins simultaneously may not enhance the phenotype associated with either single mutant. It is also possible that CycY and Eip63E function together only in specific tissues or at specific developmental times, one of which may not be embryogenesis. Thus, other phenotypic assays, perhaps using other alleles, may yet reveal genetic interactions between *CycY* and *Eip63E*.

4.3.2 *CycY*^{E8} mutants show delayed glue extrusion from salivary glands

Homozygous *CycY* mutants are lethal and have major developmental defects during metamorphosis, a process tightly regulated by ecdysone. This combined with the fact that at least one of the *Eip63E* transcripts is induced by ecdysone led me to hypothesize that CycY/Eip63E are involved in the ecdysone signaling or response pathways. To search for processes that CycY may regulate I considered ecdysone-regulated processes that are required for normal metamorphosis. One such process is synthesis of glue mixture. Glue mixture is a substance secreted by salivary glands prior to pupariation and serves to help attach the pupae to a dry surface. It is composed of several glycoproteins, one of which is sgs3. Sgs3 is expressed during the second half of third instar larval development only in the salivary glands. A few hours before

pupariation, sgs3 starts to be secreted to the salivary ducts and lumen and at puparium formation it is expelled from the salivary gland. This series of responses is under the regulation of ecdysone. A convenient tool for monitoring glue synthesis, secretion, and extrusion is a transgene, *sgs3-GFP,* which has an identical expression pattern as the endogenous *sgs3* (Biyasheva et al., 2001). I examined *sgs3-GFP* activity in *CycY* mutants. Homozygous $CycY^{E8}$ mutants showed normal glue synthesis and secretion compared to the heterozygous control (Figure 4-1 C-D compared to A-B). However, it took the mutants longer to expel the glue proteins to the outside of the salivary gland as visualized by the longer time to keep the GFP signal inside the pupae (Figure 4-1 J-M, Table 4-3). The failure to expel the secreted glue proteins to the ventral side of the body surface may be due to neural signal transduction or muscle contraction defects. The mechanism of how the loss of *CycY* may lead to delayed glue extrusion still requires further investigation. However, the identification of a specific biological process in which CycY is involved, should be useful for further delineation of CycY signaling pathways. This phenotype can be used to ask specific questions about potential upstream regulators or downstream targets. For example, from the list of potential genes that might be responsible for glue extrusion, which genes are misregulated in *CycY* mutants? Are they misregulated in *Brm* mutants too and are they direct targets of Brm complex?

4.3.3 *CycY* loss-of-function mutants show normal starvation-induced autophagic effects during the early L3 stage

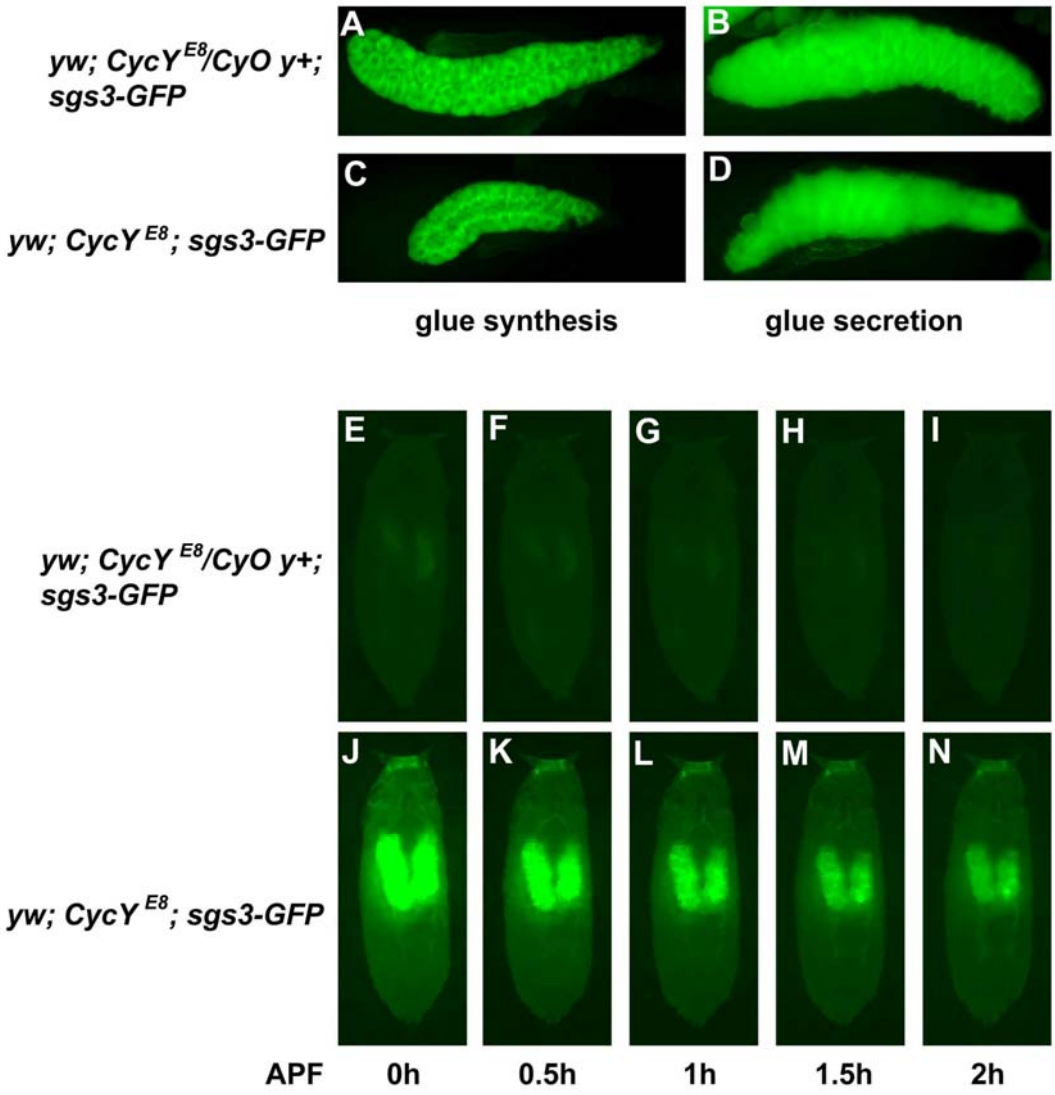


Figure 4-1. *CycY^{E8}* mutants show normal glue synthesis and secretion but delayed glue extrusion from salivary glands. (A-D) Salivary glands from late third instar larvae were dissected from heterozygous *CycY^{E8}* (A and B) or homozygous *CycY^{E8}* (C and D), which also harbored a *sgs3-GFP* transgene on the third chromosome. Glue protein was synthesized in the cytoplasm and absent from the nuclei (A and C) and then secreted to the ducts and lumen in the late third instar larvae (B and D). The glue protein-free layer of cells surrounding the lumen filled with green glue proteins is weakly visible. (E-N) Glue protein was directly visualized from synchronized prepupae at 0h (E, J), 0.5h (F, K), 1h (G, L), 1.5h (H, M), and 2h (I, N) in heterozygous *CycY^{E8}/CyO* [*y*+] (E-I) or homozygous *CycY^{E8}* (J-N), which also harbored a *sgs3-GFP* transgene on the third chromosome.

Table 4-3. Glue protein extrusion is delayed in *CycY^{E8}* mutants

Genotypes	Total pupae	Groups[a] (%)				
		1	2	3	4	5
y^{1}w*; CyO [*y+*]/*Sp; sgs3-GFP*	25	64	16	4	8	8
y^{1}w*; CycY^{E8}/CyO [*y+*]*; sgs3-GFP*	46	77	9	5	0	9
y^{1}w*; CycY^{E8}; sgs3-GFP	42	0	0	3	3	94

[a] Group 1, GFP signal almost lost at the time of puparium formation; group 2, only weak GFP signal at the time of puparium formation; group 3, medium levels of GFP signal at the time of puparium formation; group 4, strong GFP signal at the time of puparium formation, but the signal disappeared in less than 1.5h; group 5, GFP signal persisted longer than all the above mentioned situations.

Autophagy is a lysosome-mediated process of bulk cytoplasmic degradation through which long-lived proteins, organelles, and other components of the cytoplasm are engulfed within autophagosomes, the hallmark of autophagic cell death. In contrast to apoptotic cell death, cells that die with an autophagic cell death digest their own contents without the aid of engulfing phagocytes. Under starvation conditions, eukaryotic cells recover nutrients via autophagy. In addition to survival of starvation, autophagy has been implicated in many aspects of health and development, such as pathogen infection, cancer, and cell growth (Scott et al., 2007). Several lines of evidence prompted me to hypothesize that *CycY* is involved in an autophagy signaling pathway. First, *CycY* and several autophagy genes have been associated with human Crohn's disease, a complex inflammatory disease involving the small intestine affected by over 30 genetic loci (Barrett et al., 2008). Human *CycY* (*CCNY*) and the autophagy gene, *ATG16L1* and *IRGM*, were identified as susceptibility factors for Crohn's disease (Franke et al., 2008; Mizushima et al., 2003; Parkes et al., 2007; Weersma et al., 2009), suggesting that they may be involved in a common pathway. Second, autophagy is an important mechanism for eliminating obsolete larval tissues during Drosophila metamorphosis, a period when CycY is essential. Third, Snr1, a potential target of CycY (see Chapter 3), promotes the expression of autophagy genes (Zraly et al., 2006). To test whether CycY is involved in the autophagic response, I dissected well-fed and starved early L3 larvae of $CycY^{E8}$ heterozygous and homozygous mutants and stained the fat body tissue with LysoTracker Red, a lysosome-specific fluorescent dye, to detect the presence of the autophagic response. As shown in Figure 4-2, in $CycY^{E8}$ heterozygotes mutants, this response is induced by starvation in the fat body, as

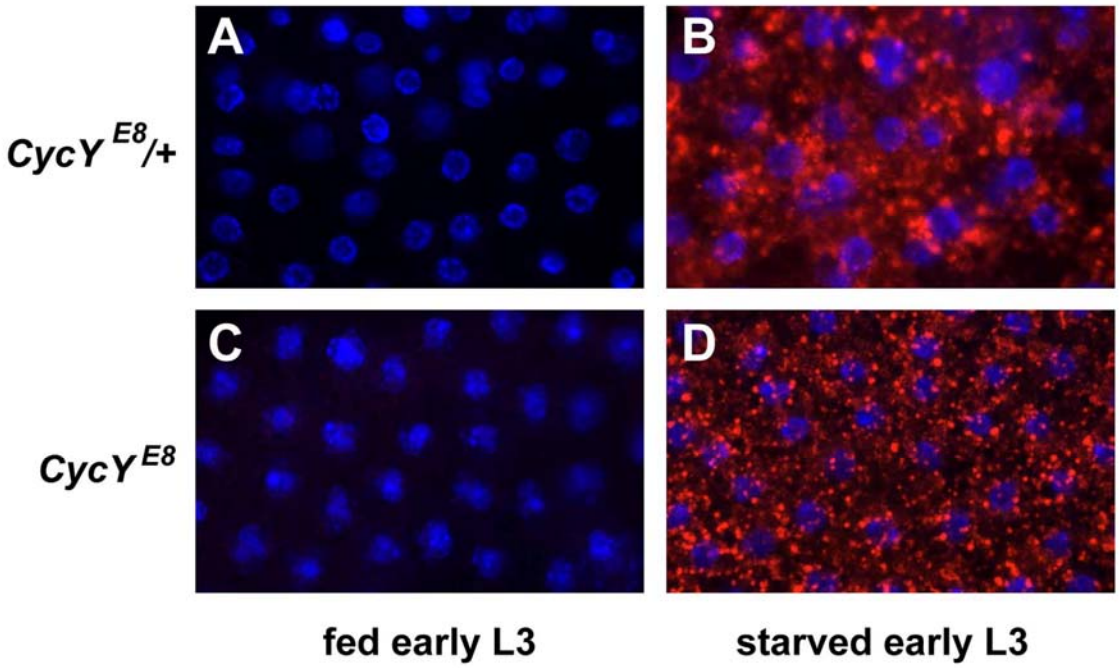


Figure 4-2. *CycY* loss-of-function mutants show normal starvation induced autophagic effects during early L3 stage. Fat bodies from well-fed (A and C) or starved (B and D) early L3 of *CycY*^{E8} heterozygotes (A-B) or homozygotes (C-D) were stained with LysoTracker (Red) and Hoechst 33342 (Blue). Fat bodies from starved larvae in both samples show induced autophagy as indicated by the appearance of lysosome staining.

expected (Figure 4-2B). Homozygous *CycY*^{E8} mutants showed a similar strong autophagic response under starvation conditions, indicating that CycY is not required for autophagy, at least not during the early L3 developmental stage (Figure 4-2D). Whether CycY is required for autophagic response during other developmental stages requires further investigation.

Autophagy is known to be inhibited by TOR (target of rapamycin) signaling and *Atg5* and *TOR* double mutants show a strong genetic enhancement of larval growth defects (Scott et al., 2004). To test whether *CycY* is involved in the autophagic response using an independent approach, I generated *CycY/Atg1* and *CycY/TOR* double mutants to look for genetic interactions. However, I was unable to detect any genetic interactions between *CycY* and *Atg1* or *CycY* and *TOR*. The larval size of *CycY/Atg1* double mutants (*CycY*^{E8}*; Atg1*$^{\Delta 3D}$) is similar to that of *Atg1*$^{\Delta 3D}$ single mutants. The same is true when comparing the larval size of *CycY/TOR* double mutants (*TOR*$^{\Delta 6B}$*; Tub-Gal4/CycYi*^{C1}) to that of *TOR*$^{\Delta 6B}$ single mutants (see Chapter 3 for details about *CycYi*^{C1}, a *CycY* knockdown transgene). Taken together, I did not detect any genetic interaction between *CycY* and *Atg1* or *TOR* as far as the phenotypes I checked.

4.3.4 The CycY expression pattern

Revealing the tissue expression pattern and subcellular localization of the *CycY* protein *in vivo* may provide clues about its biological functions. To this end, I first generated antibodies directed at a CycY peptide (see Materials and Methods) and tested the crude serum by western blot and immunostaining. However, the crude serum turned out to have high background and low sensitivity. Only high levels of exogenous

expression from a transgene could be detected, but endogenous expression could not be detected. I then tried affinity purification, but the affinity purified CycY antibody did not perform better than the crude serum. In short, the CycY antibody generated from guinea pig with a small peptide as antigen was not useful for this study. A better way to generate CycY antibodies in the future may be to immunize rabbits with purified MBP-nCycY and purify the rabbit serum, which will be available in larger quantities than serum from a guinea pig, with MBP-nCycY-coupled to beads (e.g., Affi-Gel 15).

To determine the *CycY* mRNA expression pattern, I tried RNA *in situ* hybridization. I synthesized two *CycY* probes and used them for RNA *in situ* hybridization (see Materials and Methods) of wild type and $CycY^{E8}$ mutant embryos and imaginal discs. Unfortunately, I did not detect any difference between the staining patterns in wild type and $CycY^{E8}$ mutants in either embryos or imaginal discs. This suggested that the probes do not specifically recognize *CycY*. Use of additional nonoverlapping probes in the future may overcome this problem.

An alternative approach to using antibodies to determine protein expression patterns is to use transgenes that express tagged proteins. Such an approach should be feasible for CycY. I have generated a *CycY* genomic transgene that can fully rescue the $CycY^{E8}$ null mutant to the adult stage (Chapter 2, section 2.3.3), suggesting that it may mimic the endogenous CycY function and expression pattern. This *CycY* genomic transgene includes the entire *CycY* gene and putative regulatory regions flanking the transcript, including sequences 4,032 bp upstream of the *CycY* start codon, and 1,970 bp downstream of the stop codon, and none of the coding regions of *crol* or *Pde1c* (Chapter 2, section 2.2.2). On the basis of this vector (pCaSpeR2-CycY, Finley lab #

977), a C-terminal GFP-tagged *CycY* genomic vector could be generated by regular molecular cloning approaches. For example, there are two unique restriction enzyme digestion sites upstream and downstream of the *CycY* TGA stop codon, AvrII and MfeI respectively. By regular PCR, three fragments can be amplified, from AvrII to the last codon of *CycY*, ORF of GFP tag, and from *CycY* stop codon TGA to MfeI genomic region. By including suitable restriction enzyme digestion sites at each end of the PCR products, these three fragments could be sequentially cloned into a cloning vector. PCR of the three ligated fragments would generate a fragment that could replace the original AvrII/MfeI fragment in pCaSpeR2-CycY by gap repair or by restriction digestion and ligation. In this modified vector, a C-terminal GFP tag would be added just in front of the *CycY* TGA stop codon. Transgenic flies could then be generated and used to determine the spatial, temporal expression pattern, and subcellular localization of CycY by following GFP fluorescence signal.

4.3.5 Subcellular localization of a complementing myc-CycY fusion protein

Two recent publications about human CycY (CCNY) in cultured cells independently concluded that the N-terminus of CycY has a myristoylation signal, which anchors the protein to the cell membrane. Mutation of the putative myristoylation site, glycine 2, to an alanine abolished membrane localization in cultured cells, as did fusion to N-terminal tags such as GFP or HA. Forced expression of CycY resulted in relocalization of GFP-PFTK1 from the cytoplasm to the plasma membrane, indicating that this cyclin is capable of recruiting the Cdk to the membrane (Davidson et al., 2009; Jiang et al., 2009). The presence of a myristoylation signal and the localization of CycY

to the plasma membrane make it likely that CycY functions at least in part by localizing Cdk activity to the plasma membrane to direct phosphorylation of membrane-associated substrates. One such substrate identified by Davidson et al is LRP6, a Wnt co-receptor (Davidson et al., 2009). Their data suggest that phosphorylation of LRP6 by CycY/PFTK1 primes the subsequent Wnt-dependant phosphorylation and hence activates the Wnt signaling pathway. They showed that mutation of the myristoylation signal of CycY decreased the PFTK1-dependent phosphorylation of LRP6. The potential importance of the membrane localization and myristoylation of CycY function in human cells raises the question of whether it is also important in Drosophila. Due to the lack of a good anti-CycY antibody, I tested the subcellular localization of an N-terminally myc-tagged CycY expressed in Drosophila S2R+ cells. Consistent with the data with human cells, in Drosophila cells CycY with an N-terminal myc tag is mainly localized in the cytoplasm, as is myc-Eip63E (Figure 4-3). I surmise that the N-terminal myc tag is blocking membrane localization; however, I have not tried a C-terminally tagged CycY to verify that the lack of an N-terminal tag would result in membrane localization. This experiment should be done to see where native CycY localizes. In the meantime, results with the myc-tagged CycY suggest that membrane localization may not be very important for *in vivo* function. Evidence for this comes from complementation studies I conducted with myc-CycY. I generated transgenic flies expressing myc-tagged CycY from a cDNA, under control of either UAS or the heat shock promoter. Myc-CycY expressed from the heat shock promoter or using a ubiquitous Gal4 driver line rescued $CycY^{E8}$ null mutants to the adult stage, suggesting that myc-CycY has no obvious functional defects, at least in Drosophila. Combined with

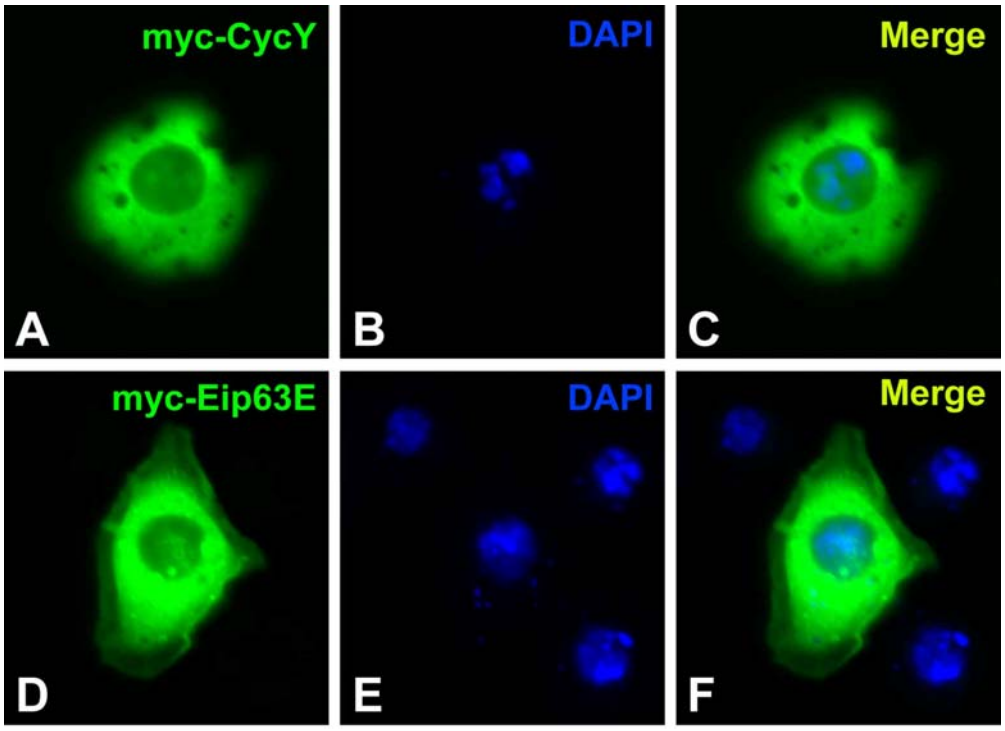


Figure 4-3. The subcellular localization of N-terminal myc-tagged CycY and Eip63E. Drosophila S2R+ cells expressing myc-CycY (A-C) or myc-Eip63E (D-F) were stained with myc Ab (green, A and D) and DAPI (blue, B and E).

the fact that N-terminal tags can disrupt membrane localization and we observed no membrane localization for myc-CycY, these data suggests that membrane anchoring is not important for CycY function in Drosophila. Alternatively, overexpression of myc-CycY *in vivo* may bypass the requirement for membrane localization. Further analysis of the requirement for a myristoylation signal *in vivo* will be required to resolve this question.

The cytoplasmic localization and full rescue ability of myc-CycY bring up more interesting questions. Does CycY/Eip63E complex also have an active role in Wg signaling transduction in Drosophila as in human and Drosophila cells? Is the membrane localization of CycY essential to fulfill this function in Drosophila? How does loss of CycY *in vivo* influence Wg signaling? If the membrane-anchored CycY is essential for Wg signaling transduction, then can we explain the full rescue ability of myc-CycY? One possibility is that a small amount of myc-CycY is anchored to the membrane and this is sufficient for LRP6 phosphorylation and Wg pathway function *in vivo*. Another possibility is that there are other redundant cyclins to replace CycY at least for phosphorylation of arrow/LRP6 or that this phosphorylation can be fulfilled by other known arrow/LRP6 kinase, sgg/GSK3, *in vivo*. Finally, it is possible that arrow/LRP6 phosphorylation by a CycY kinase is only one of several CycY functions and that it is not essential for viability. To distinguish these possibilities and to gain a better understanding of the importance of membrane localization for *in vivo* CycY function, the generation of *CycY*^{M2A} mutant transgenic flies seems necessary.

It is interesting to note that the N-terminally truncated isoform of human CycY, which lacks the myristoylation signal, has been shown to be predominantly localized in

the nucleus. It has also been shown to activate the transcriptional activities of c-Myc (Li et al., 2009), which is one of the downstream regulatory targets of Wnt/β-catenin signaling (He et al., 1998). Does this suggest that the short isoform of CycY, which is not a membrane anchored protein, also regulates Wnt signaling by phosphorylating LRP6? Alternatively, it is possible that the short isoform of CycY functions in the nucleus to modulate the transcriptional activities of myc.

4.3.6 The CycY/Eip63E complexes expressed in Drosophila cells is unable to phosphorylate Snr1 *in vitro*

Since Snr1 physically interacts with CycY *in vitro* and the molecular function of the cyclin/Cdk complexes is to phosphorylate downstream targets, a simple hypothesis to explain the genetic interaction between CycY and the Brm complex is that Snr1 is the substrate of CycY/Eip63E. To test this hypothesis, I expressed and purified MBP-Snr1, MBP-Snr1 T102A, MBP-Snr1 T198A, and MBP-Snr1 T102/198A fusion proteins from *E.coli* and used them as substrates for *in vitro* kinase assays using CycY/Eip63E expressed in Drosophila S2R+ cells. I expressed myc-tagged CycY, Eip63E, or both and various controls, then affinity purified the tagged fusion proteins and used them for *in vitro* kinase assays as previously described (Kolonin and Finley, 2000) (Guest et al. in prep.). The results provided no evidence for specific phosphorylation of Snr1 by CycY/Eip63E (Figure 4-4). Although MBP-Snr1 appeared to be more strongly phosphorylated by purified myc-Eip63E than by the control (lane 2 vs. lane 9), the signal was the same with the myc-Eip63E kinase-dead mutant (lane 2 vs. lane 4). Moreover, Snr1 phosphorylation was not strengthened in the presence of CycY (lane 2 vs. lane 6).

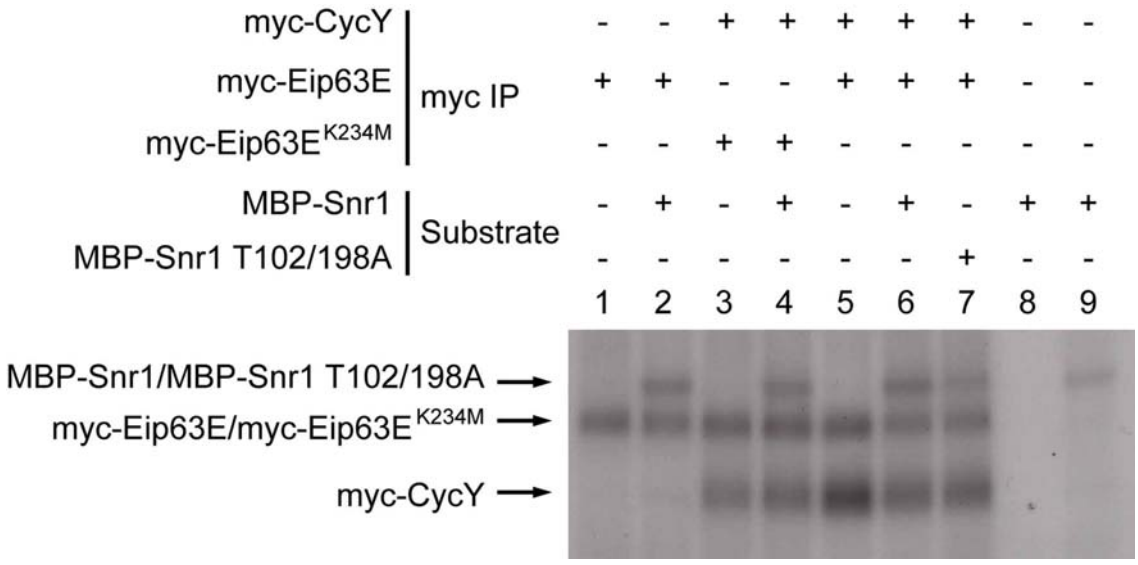


Figure 4-4. The CycY/Eip63E complex is unable to phosphorylate Snr1 *in vitro*. Drosophila S2R+ cells were cotransfected with the indicated constructs and lysed for pull down with myc antibody. γ^{32}P [ATP] was added along with maltose binding protein (MBP) fusion proteins purified from *E.coli* expression as indicated to each immunoprecipitate as *in vitro* substrates. Both lane 8 and 9 are negative controls. Lane 8 had no immunoprecipitate, while lane 9 had anti-myc immunoprecipitate from untransfected cells.

These results indicate that MBP-Snr1 is weakly phosphorylated by a kinase that co-immunoprecipitates with myc-Eip63E. The relatively weak phosphorylation signal from MBP-Snr1 T102/198A suggests that threonine 102 or 198 are among the phosphorylation sites (lane 6 vs. lane 7).

One possible explanation for our failure to detect Snr1 phosphorylation by CycY/Eip63E is that some coactivators of the Eip63E kinase may be missing from the cultured cells or the immunoprecipitates. For example PIF-1B is an Eip63E-interacting protein that has been proposed to counter the inhibitory effect of the long N-terminal extension of Eip63E (Rascle et al., 2003). We do not know whether PIF-1B is expressed in the S2R+ cells. Another possible explanation for the kinase assay results is that Snr1 may not be a direct substrate of CycY/Eip63E and may function as a bridge to bring CycY/Eip63E to the vicinity of the Brm multi-protein complex to phosphorylate other components. The possible CycY-dependent phosphorylation of other components in the Brm complex should be tested in the future.

4.3.7 *CycY^{E8}* null mutant clones do not show defective cell growth or proliferation in third instar larvae imaginal discs and fat bodies compared to wild type sister clones

Genetic mosaic techniques provide a way to examine a lethal homozygous mutation in just a subset of cells *in vivo*. To look for developmental defects associated with loss of *CycY* from sets of cells, I generated *CycY^{E8}* null clones either specifically in the eye with ey-FLP or in all developing tissues with hs-FLP by FRT-mediated mitotic recombination (Xu and Rubin, 1993). Consistent with the normal eye of *CycY^{E8}* adult

escapers or pharate adults (Liu and Finley Jr, 2010), the eye of the adults with *CycY*^{E8} null clones also look normal (data not shown). To check the *CycY*^{E8} null clones induced by ubiquitous FLP, third instar larvae with yellow mouth hooks (*y*^{1}*w** *hs-FLP; CycY*^{E8} *FRT40A/Ubi-GFP FRT40A*) were dissected. The GFP+ cells (*CycY*^{E8}*/Ubi-GFP* or *Ubi-GFP/Ubi-GFP*) and the GFP- cells (*CycY*^{E8}) from the imaginal discs and fat bodies were compared. Surprisingly, I did not find any defective cell growth or proliferation when comparing the size of *CycY*^{E8} null clones with sister twin clones or the individual cell size of *CycY*^{E8} null and wild type (Figure 4-5 A-R). In addition, after staining for phosphorylated histone H3 (PH3) to visualize cells undergoing mitosis, I observed no obvious difference between wild type and *CycY*^{E8} null clones (Figure 4-5 S-U). I also did not detect any adult morphological defects. These results are unexpected and hard to explain considering that zygotic *CycY*^{E8} null mutants had delayed larval growth (delayed 13 hours compared with the heterozygous control animals), small sized pupae (90% compared with the heterozygous control), and pupal lethality (Chapter 2, section 2.3.3). One possible explanation is that both the delayed larval growth and the decreased pupal size phenotypes are not very strong, therefore the mild difference between *CycY*^{E8} null clones and wild-type sister twin clones is not easily appreciated. Another possibility is that these *CycY*^{E8} null clones were formed during larval development and the *CycY* proteins synthesized before the null clone formation lasted long enough beyond the identification of any visible effect.

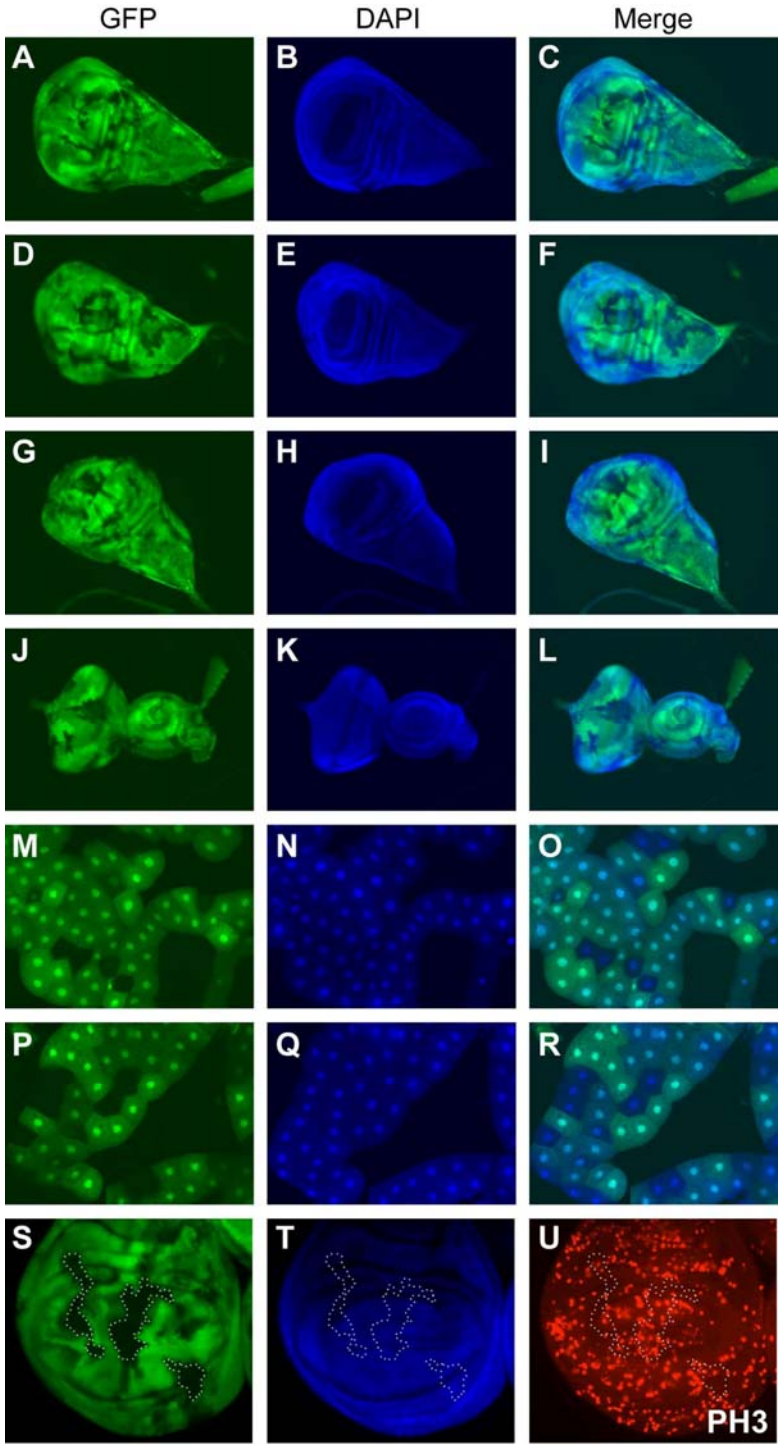


Figure 4-5. *CycY*^{E8} null mutant clones do not show defective cell growth or proliferation in third instar larvae imaginal discs and fat bodies. Third instar larvae with yellow mouth hooks (*y*^{1}*w** *hs-FLP; CycY*^{E8} *FRT40A/Ubi-GFP FRT40A*) were dissected and stained with DAPI (blue). *CycY*^{E8} null clones are GFP negative. Representative wing imaginal discs (A-I, S-U), eye imaginal disc (J-L), and fat bodies (M-R) are shown. White dotted line circles the *CycY*^{E8} null clones in the wing imaginal disc (S-U). The existence of *CycY*^{E8} null clones suggests that absence of *CycY* is not cell lethal.

4.4 Conclusions

In this chapter, I showed that *CycY* does not genetically modify the hatching rate defect associated with $Eip63E^{81}$ allele, which neither supports nor weakens my hypothesis that CycY and Eip63E form an *in vivo* cyclin Cdk pair. I also showed that *CycY* null mutants display normal starvation induced autophagic effects during the early third instar larval stage. Mosaic analysis of *CycY* null clones did not reveal any defective cell growth or proliferation in third instar larvae imaginal discs and fat bodies compared to wild type sister clones. Interestingly the process of glue extrusion, which is regulated by ecdysone, is misregulated in the *CycY* mutant. This developmental process could be used in the future to help elucidate the signaling pathways to which *CycY* belongs. Due our failure to generate high-quality CycY antibodies and to the non-specific RNA *in situ* hybridization, I was unable to determine the endogenous *CycY* protein or mRNA localization. However, consistent with studies with human cells, I showed that CycY with an N-terminal myc tag is mainly localized in the cytoplasm. Intriguingly, this version of CycY successfully rescued the $CycY^{E8}$ null mutant to the adult stage, raising the questions of the importance of the membrane localization of CycY and of its role in Wg signaling *in vivo*. Finally, *in vitro* kinase assays suggest that Snr1 may not be a direct phosphorylation target of the CycY/Eip63E complex. This result suggests that Snr1 may function as a bridge to bring CycY/Eip63E to the vicinity of the Brm complex to phosphorylate other components and therefore regulate the Brm complex activity.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

In this dissertation, I described the characterization of a novel conserved cyclin *CG14939* (*CycY*) in Drosophila. I generated the first mutant animal model for this gene as well as transgenic RNAi strains that can be used to specifically knock down the expression of *CycY* in a temporally and spatially controlled manner. I provided multiple lines of evidence showing that the Cdk partner of CycY is Eip63E (Cdk14). I also showed that *CycY* is an essential gene that is required from oogenesis to adult viability. Finally, I demonstrated the physical and genetic interaction between CycY and components of Brm complexes from several different aspects. Here I summarize some of the major findings and implications.

5.1.1 *CycY^{E8}* is a null mutant allele of *CycY*

Using P-element imprecise excision, I created a *CycY* null mutant allele, *CycY^{E8}*, which lacks all *CycY* coding sequences. Multiple lines of evidence indicate that *CycY* is the only gene affected in strain *CycY^{E8}*. First, the two neighboring genes, *crol* and *Pde1c,* are expressed in homozygous *CycY^{E8}* larvae, in contrast to *CycY*. Second, *CycY^{E8}* and *crol^{04418}*, a lethal null allele of the neighboring gene, mutually complemented the mutant phenotypes of each other, suggesting that *crol* is fully functional in *CycY^{E8}*. Third, transheterozygous mutants of *CycY^{E8} over* a deficiency

strain that lacks *CycY* (*Df(2L)Exel6030*), display similar developmental defects to the homozygous $CycY^{E8}$ mutants. Fourth and definitively, a *CycY* genomic transgene or ubiquitous expression of a *CycY* cDNA can substantially rescue most of the abnormalities that I observed in homozygous $CycY^{E8}$ mutants, including the delayed larval growth, defects during metamorphosis, and adult viability. However, I have not yet tested whether the $CycY^{E8}$ oogenesis defect and embryonic lethality can be rescued with *CycY* transgene, so it is formally possible that a second site mutation may partially or fully contribute to these. A rescue experiment using the maternal and zygotic $CycY^{E8}$ null mutants will be necessary for any future study of the early developmental requirement of CycY. The $CycY^{E8}$ mutant strain provides a powerful tool for further functional characterization of CycY in Drosophila.

5.1.2 $CycYi^{C}$ can be used to specifically knock down the expression of *CycY*

In this project, I generated multiple transgenic fly strains for each of two non-overlapping RNAi constructs, $CycYi^{N}$ and $CycYi^{C}$. Multiple lines of evidence suggest that $CycYi^{C}$ can be used to specifically knock down the expression of *CycY*. Expression of $CycYi^{C}$ with the *en-Gal4* driver led to decreased wing size in the posterior compartment where *CycY* expression is reduced compared with the anterior region with wild-type *CycY* expression. This decreased wing size phenotype can be partially rescued by expression of a *CycY* cDNA, suggesting that the observed phenotype was due to decreased expression of *CycY* and not an off-target effect. Expression of $CycYi^{C}$ with the *69B-Gal4* driver induced extra wing vein tissue, which can be fully rescued by expression of a *CycY* cDNA, again indicating a *CycY*-specific knockdown effect. A final

piece of evidence for the specific knockdown ability of *CycYi*^{C} comes from the genetic interaction data. Using the *69B-Gal4* driver to knock down *Brm* resulted in no obvious defects. However, combining *Brm* knockdown with either *CycYi*^{C} or the removal of one copy of wild-type *CycY* led to a novel wing phenotype, suggesting not only a genetic interaction between *Brm* and *CycY*, but also the specificity of the *CycY* knockdown. Combined, these results suggest that *CycYi*^{C} specifically knocks down the expression of *CycY*. The fly strains harboring the *CycYi*^{C} transgene will be very useful in future studies to test for tissue specific requirements of CycY and to help identify the signaling pathways to which CycY may belong. For each new phenotype detected with *CycYi*^{C}, a test for specificity should be conducted. For example, the *CycYi*^{C} phenotype should be tested to see if it is enhanced by *CycY*^{E8} or suppressed by a *CycY* cDNA.

5.1.3 The Cdk partner of CycY is Eip63E/PFTK1/Cdk14

A potential Cdk partner for CycY was first identified in a large-scale yeast two-hybrid screen with Drosophila proteins (Stanyon et al., 2004). The identified kinase was Eip63E, which previously had no known cyclin partner (Rascle et al., 2003; Stowers et al., 2000). I demonstrated that CycY and Eip63E specifically interact when expressed with N-terminal affinity tags in Drosophila S2R+ cells. However, due to the lack of antibodies to either CycY or Eip63E, I was unable to perform *in vivo* pull-down assays to test whether they are *bona fide in vivo* partners. Nevertheless, several additional lines of evidence support this conclusion. If Eip63E and CycY form a functional Cdk/cyclin complex *in vivo*, we might expect their mutant phenotypes to be similar. Therefore I performed a detailed side-by-side phenotypic characterization of *CycY* and *Eip63E* null

mutants and showed that they are strikingly similar. Further supporting evidence that CycY regulates Eip63E comes from studies in human cells. The human ortholog of Eip63E is PFTK1. Two recent publications about human CycY (CCNY) in cultured cells independently demonstrated the PFTK1/CycY interaction by co-AP assays and showed that membrane-anchored CycY recruits cytoplasmic PFTK1 to the membrane (Davidson et al., 2009; Jiang et al., 2009). Davidson et al further showed that Wnt co-receptor LRP6 is a membrane-associated phosphorylation target of the CycY/PFTK1 complex (Davidson et al., 2009).

An additional argument for the Cdk14/CycY partnership comes from examination of sequence conservation. Cyclin proteins are present in non-metazoans but they do not share any extensive sequence similarity to CycY. Some of the non-metazoan cyclin proteins, however, do possess a CycY-specific cyclin box (Liu and Finley Jr, 2010). This domain is similar to the CDK-binding domain in other cyclins but it appears to have emerged in a common ancestor of plants and animals. The Y–type cyclin box in yeast or plant cyclins, for example, is more similar to the same domain in human and Drosophila CycY than to the other yeast or plant cyclin boxes, suggesting that this domain is ancient and has been conserved independently of the other cyclins over 500 million years of evolution. A specific example of this conservation can be seen in the yeast cyclin PCL1. This protein is the reciprocal best-matched protein for both human and Drosophila CycY; this means that PCL1 is the yeast protein most similar to human or Drosophila CycY, which are in turn the human and Drosophila proteins most similar to PCL1. Essentially all of this sequence similarity resides in the CycY-specific cyclin box. The Cdk partner for PCL1 is Pho85, which has a number of cyclin regulators and

plays diverse roles in yeast (Huang et al., 2007). In the context of establishing the Cdk partner for CycY, it is interesting to note that the reciprocal best-matched protein for Pho85 in human and Drosophila is PFTK1 and Eip63E, respectively. This raises the intriguing possibility that this particular cyclin-Cdk interaction is ancient and may have novel properties that distinguish it from other cyclin-Cdk pairs. Taken together, these data suggest that CycY and Cdk14 constitute a conserved cyclin-Cdk pair.

5.1.4 *CycY* is required from oogenesis to adult life

A thorough characterization of $CycY^{E8}$ null mutant phenotypes revealed that CycY plays an essential role during almost all developmental stages. The $CycY^{E8}$ zygotic null mutant is lethal with most mutant animals arresting during pupal development. The mutant exhibited delayed larval growth and major developmental defects during metamorphosis, including impaired gas bubble translocation, head eversion, leg elongation, and adult tissue growth. Heat-shock-induced expression of *CycY* at different times during development resulted in variable levels of rescue, the timing of which suggests a key function for zygotic *CycY* during the transition from third instar larvae to prepupae. I further took advantage of the availability of the heat-shock rescued $CycY^{E8}$ mutant adults and found that *CycY* is required for adult longevity, especially in males. Using the ovo^{D1} dominant female sterile technique, I revealed that maternal CycY also plays a role during oogenesis and is required for embryogenesis; interestingly, this role can be accomplished at least to a limited extent by zygotic expression.

By testing the tissue specific knockdown of *CycY* and genetic interaction between CycY and Brm complex components, I was able to show a few tissue specific requirements of CycY. Knockdown of *CycY* with *en-Gal4* and *69B-Gal4* drivers revealed that CycY is involved in wing growth and wing vein development. Removal of one copy of *CycY* enhanced the abdominal dorsal midline fusion defect associated with *Snr1* mutants, suggesting a role of CycY in the development of the integument of the adult abdomen. The identification of the tissue- and developmental stage-specific requirements of CycY provides a platform through which the biological functions of CycY can be further elucidated.

5.1.5 *CycY* genetically and physically interacts with components of the Brm complex and is involved in regulation of Brm complex target genes

To gain insight into the cellular functions of CycY and to identify the signaling pathways to which CycY belongs, I began by examining the available protein interaction data for CycY and Cdk14. Among all the identified and predicted physical interactors, Snr1 shares similar developmental requirements and expression profiles with CycY. To test whether CycY and Snr1 belong to a common signaling pathway, I first confirmed the physical interaction between CycY and Snr1 by co-AP assay from cultured cells. I further showed that CycY and two Brm complex components, Snr1 and Brm, genetically interact during multiple developmental processes. For example, removal of one copy of *CycY* enhances the adult longevity and dorsal midline fusion defects induced by expression of truncated *Snr1* in the heterozygous *Snr1* null background. Knockdown of *CycY* in the wing enhances the extra wing vein phenotype induced by *Snr1* knockdown,

as well as the wing phenotype induced by *Brm* knockdown or overexpression of a *Brm* dominant negative mutant. The genetic interaction between CycY and members of the Brm complex, combined with the physical interaction between CycY and Snr1, suggests that CycY may promote Snr1 functions in the Brm complex to regulate gene expression in a cell-type specific manner. The examination of gene expression of a group of Brm complex targets, the *Eig71E* genes, however, suggested that CycY and Snr1 may also regulate gene expression differentially in a temporal-specific manner. This led to a model in which CycY promotes the activity of Snr1, which can either activate or repress transcription of specific genes depending on the time and tissue (Fig. 3-10).

This is the first identification of a signaling pathway in which CycY may be involved in Drosophila. It helps explain some of the developmental defects that *CycY* null mutants displayed. However, whether the genetic interactions I observed are due to direct regulation of Brm complex activity by CycY or due to a function of CycY in a parallel signaling pathway is still unclear. My hypothesis is based on the assumption that CycY physically interacts with Snr1. However, I was unable to test this putative interaction *in vivo* due to lack of antibodies. Furthermore, I failed to show that Snr1 is a direct phosphorylation target of CycY/Cdk14 complex *in vitro*. The identification of the involvement of CycY in Wnt signaling in cultured human and Drosophila cells also suggested another way to interpret the genetic interaction between CycY and Snr1/Brm (see section 5.2.1). Testing for *in vivo* interactions between CycY and other Brm complex components may help clarify these issues.

5.1.6 Model for CycY function

Based on data from this study and from others, I propose the following model for CycY functions (Figure 5-1). CycY may have different subcellular localizations that recruit Cdk14 to the vicinity of different substrates. Cytoplasmic or nuclear CycY/Cdk14 complexes may phosphorylate Snr1 or other components of the Brm complex to regulate genes required at many developmental stages, including metamorphosis. Membrane-anchored CycY may recruit Cdk14 to phosphorylate membrane substrates, such as the Wg co-receptor Arrow. The phosphorylation of Arrow activates Wg signaling, which in turn regulates cell proliferation, differentiation, and pattern formation in a variety of developmental contexts. Although each of the two putative CycY/Cdk14 substrates, Brm complex components and Arrow, could help explain some of the phenotypes of *CycY* mutants, neither of them has been confirmed to be a *bona fide in vivo* substrate. Even the partnership of CycY and Cdk14 has not been definitively determined *in vivo*. Thus further studies to identify the precise *in vivo* Cdk binding partner and substrates are required.

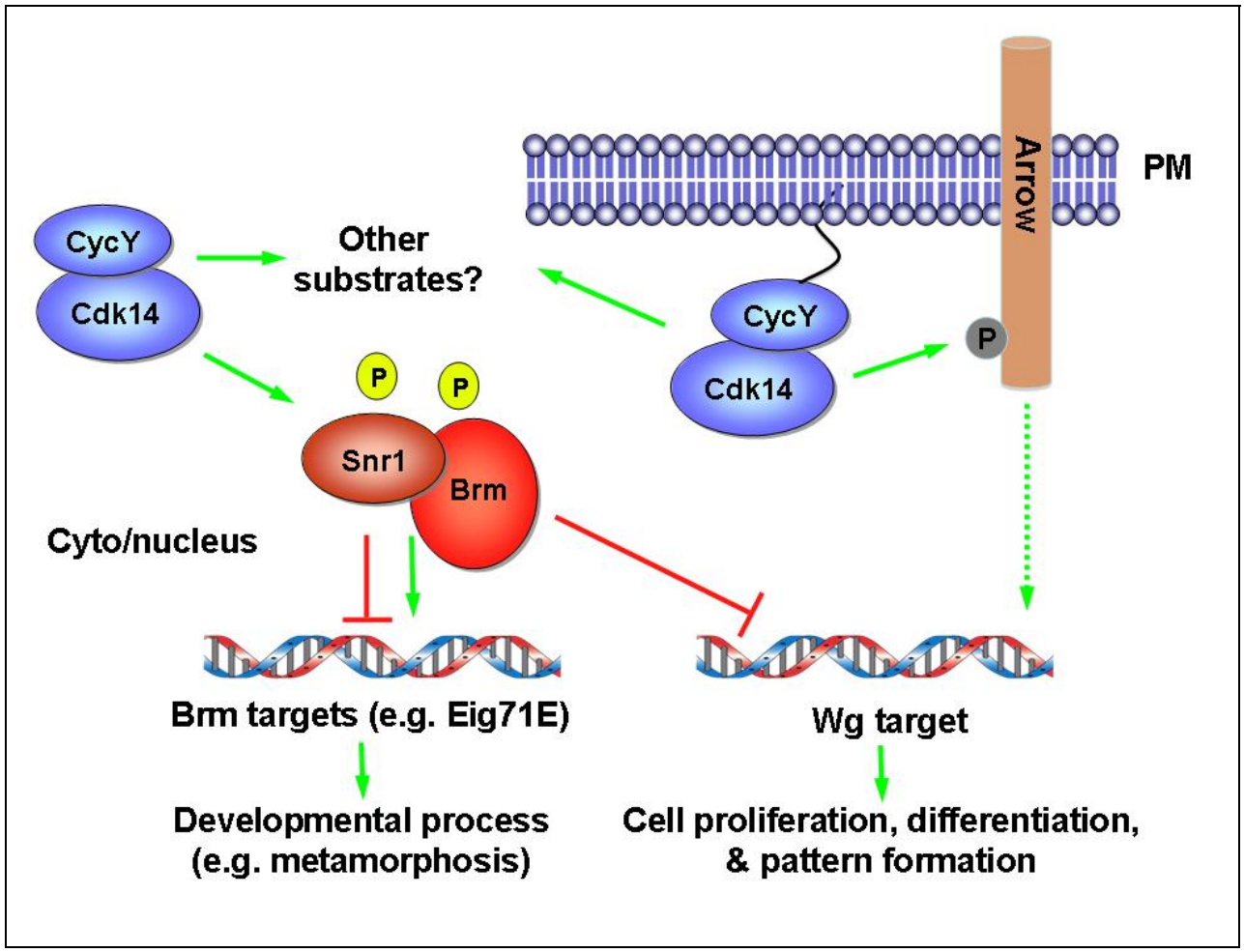


Figure 5-1. Model for CycY function. CycY may have different subcellular localizations that recruit Cdk14 to the vicinity of different substrates. Cytoplasmic or nuclear CycY/Cdk14 complexes may phosphorylate Snr1 or other components of the Brm complex to regulate genes required at many developmental stages. The regulation of *Eig71E* genes, for example, may partially contribute to the regulation of metamorphosis. CycY can also be tethered to the plasma membrane (PM) via an N-terminal myristoylation signal. CycY recruits Cdk14 to the membrane where it phosphorylates the Wg co-receptor, Arrow, primarily during G2/M phase of the cell cycle. The phosphorylation of Arrow activates Wg signaling, which in turn regulates cell proliferation, differentiation, and pattern formation in a variety of developmental contexts. The Brm complex has been directly linked with Wg signaling through inhibition the expression of Wg targets. Other substrates and roles for CycY/Cdk14 have yet to be identified.

5.2 FUTURE DIRECTIONS

Data presented in this dissertation represent an initial functional characterization of *CycY*. Many interesting *CycY* mutant defects were identified and described. The potential Cdk interacting partner and potential downstream regulatory targets were also revealed. However, the molecular mechanisms behind the role of *CycY* during these developmental processes are still to be elucidated. The following questions must be addressed to gain a better understanding of CycY functions.

5.2.1 Does CycY/Eip63E play a role in Wg signaling in Drosophila?

It has been reported recently that in both human and Drosophila cultured cells, membrane-anchored CycY recruits its Cdk partner, Cdk14, to the cell membrane to phosphorylate a membrane substrate, the Wnt/Wg co-receptor LRP6/Arrow. This was proposed to prime subsequent phosphorylation of LRP6 by CK1γ and thereby activate canonical Wnt signaling (Davidson et al., 2009; Jiang et al., 2009). However, this pathway has not been tested *in vivo* in Drosophila. Moreover, the phenotypes of *CycY* mutants are not typical of Wg pathway mutants. In Chapter 2 and Chapter 3, I showed that *CycY* is required for embryogenesis, larval growth, metamorphosis, and adult viability. Thus, it seems likely that at least some of the *CycY* phenotypes are due to defects in signaling pathways that are independent of Wg signals.

Further genetic analysis will be necessary to test whether CycY/Eip63E is involved in Wg signaling *in vivo*. The tools generated in this study will be useful for this genetic analysis. For example, a well-known defect of Wg signaling pathway mutants is disrupted segmental patterning. The Drosophila embryonic ventral epidermis is

composed of two types of epidermal cells, denticle-secreting cells, which create short, thick hair-like structures, and smooth cuticle-secreting cells. Denticle-secreting cells form segmentally repeated belts separated by smooth-cuticle belts, the pattern of which is determined by the activation of several signaling pathways during embryogenesis, including Wg, Hedgehog, EGF, and Notch signaling pathways (Alexandre et al., 1999; Gritzan et al., 1999). Wg signaling is active in smooth cuticle-secreting cells, while inactive in denticle-secreting cells. Therefore, mutants of positive regulators of Wg signaling, such as *arrow*, *wls*, and *Wg*, show ectopic denticles, while mutants of negative regulators, such as *bili* and *sgg,* show ectopic smooth cuticles (Banziger et al., 2006; Bejsovec and Wieschaus, 1993; Kategaya et al., 2009; Siegfried et al., 1992; Wehrli et al., 2000). Analyzing the ventral cuticle patterning, therefore, is a useful way to detect defects in Wg signaling (Alexandre, 2007). I have shown that a maternal and zygotic null of $CycY^{E8}$ is embryonic lethal. However, no further analysis has been performed. The cuticle patterning deserves to be carefully examined in the future to see if *CycY* mutants display defects associated with disrupted Wg signaling.

Another major morphological defect in mutants of Wg pathway components is the loss of wing or wing margin. For example, blocking Wg signaling at the wing margin by expressing a constitutively active form of Sgg causes a loss of the wing margin tissue (Collins and Treisman, 2000). Knocking down *Wg* or *wls* by dsRNA expressed under *en-Gal4* control induces the loss of wing margin specifically in the posterior compartment of the wing (Boutros and Ahringer, 2008). Knockdown of *CycY* by *en-Gal4*/RNAi, however, led to decreased wing size in the posterior region without generating a wing margin defect. This suggests that CycY has functions beyond Wg

signaling, even if it does regulate Wg signaling in some tissues. Further testing for genetic interactions between *CycY* and Wg signaling mutants should help establish whether and to what extent CycY plays a role in Wg signaling.

I have shown that CycY genetically interacts with the Brm complex components, Snr1 and Brm. Interestingly, the Brm complex has been found to inhibit the expression of Wg targets (Collins and Treisman, 2000). This situation makes the interpretation of the genetic interactions between CycY, the Brm complex, and potential Wg pathway components difficult. The genetic interaction between CycY and the Brm complex, for example, could be explained by the action of CycY on the Brm complex or on the Wg pathway, or both. To distinguish these possibilities, the epistatic relationship between CycY, the Brm complex, and Wg targets should be investigated. If CycY functions in Wg signaling to regulate Wg target expression, it should be upstream of the Wg pathway transcription factor, β-catenin/Arm. If CycY functions through the Brm complex to indirectly regulate Wg target genes, it should be downstream of Arm. One can test the expression of Arm protein and Arm target genes, such as *dll* by utilizing a dll-LacZ fly strain, in *CycY* mutant to determine the epistatic relationship between them.

5.2.2 What is the significance of the myristoylation signal and presumptive membrane localization to CycY function?

Human CycY has been reported to have two isoforms, one with and one without the N-terminal myristoylation signal, which is required to anchor CycY to the cell membrane (Jiang et al., 2009; Li et al., 2009). The subcellular localization of endogenous Drosophila CycY has not been tested either *in vitro* or *in vivo*, although the

myristoylation signal does exist. Whether there is a non-membrane anchored isoform of Drosophila CycY has not been determined. To identify the existence of other isoforms of *CycY*, it will be useful to perform a Northern blot with a probe that recognizes the 3' end of the *CycY* transcript. To test the subcellular localization of CycY *in vivo*, I have suggested in Chapter 4 (section 4.3.4) generation of transgenic flies with a *CycY* genomic transgene, into which a C-terminal GFP or myc tag should be inserted. The reason that a C-terminal tag must be used is that N-terminal tags block the myristoylation signal and the membrane localization in human CCNY. To test localization *in vitro*, similarly, *CycY* cDNA should be cloned into a cell culture expression vector with a C-terminal tag, for example pMK33-CTAP (Finley lab # 840).

The N-terminal myristoylation signal of CycY has been shown to be crucial for recruiting Cdk14 to the membrane to phosphorylate LRP6 and activate Wnt signaling (Davidson et al., 2009). Is the membrane localization essential for all or part of the CycY functions in Drosophila? At this point, we do not know the subcellular localization of endogenous CycY, but consistent with data from human cells, an N-terminally tagged CycY localizes primarily to the cytoplasm. Interestingly, this form of CycY (myc-CycY) can successfully rescue the $CycY^{E8}$ null mutant into the adult stage. This would seem to suggest that membrane localization and arrow phosphorylation are not necessary for CycY function *in vivo*. However, it is possible that a small fraction of myc-CycY spends some time attached to the membrane to recruit Cdk14 and phosphorylate arrow. It is also possible that the sgg kinase is sufficient to phosphorylate arrow in the absence of membrane-anchored CycY. To distinguish between these possibilities, I suggest generating transgenic flies that harbor genes to express either truncated CycY, which

lacks the N-terminal myristoylation signal, or a mutated CycY, $CycY^{M2A}$, that should not localize to the membrane, and to test how well these flies rescue the $CycY^{E8}$ null mutants. Such transgenic flies could also be used in genetic interaction assays with Wg pathway mutants to gain further insight into the relationship between CycY, membrane localization, and the Wg pathway.

5.2.3 Is CycY involved in the chromatin modification mediated by the Brm complex?

In this project, I identified genetic interactions suggesting that CycY and components of the Brm chromatin-remodeling complex function together to regulate multiple developmental processes. However, the molecular mechanisms that account for these interactions are still unknown. The physical interaction between CycY and Snr1 prompted me to hypothesize that CycY, together with its Cdk partner Cdk14, may phosphorylate Snr1 or other components of the Brm complex to regulate the activity of ATP-dependent chromatin remodeling. Several experiments could be used to test this hypothesis. First, one could test whether CycY interacts with other components of the Brm complex by Y2H or co-AP assays from fly cells. Ideally any interactions detected should be confirmed *in vivo* by pull-down assays; however, this will require antibodies against CycY and Brm complex components. Second, it would be helpful in understanding the function of the CycY-Snr1 interaction if one could refine the physical interaction between them. A mutagenic PCR approach could be used to identify a CycY mutant unable to interact with Snr1, but still able to interact with Eip63E. This mutant form of CycY could then be used to test how well it mimics wild type CycY. Third, it

would be useful to establish an *in vitro* assay system to test the influence of CycY on transcription of Brm complex targets. For example, *stg* transcription is directly regulated by the Brm complex in Drosophila S2 cells. Several questions should be addressed. For instance, how is the transcription of *stg* regulated in the absence of CycY? Does the Brm complex still bind to the *stg* promoter in the absence of CycY? These questions can be answered by doing RT-qPCR of *stg* and ChIP with Brm Ab.

5.2.4 What other signaling pathways is CycY/Cdk14 complex involved in?

The work described in this dissertation and from another group suggested that CycY may be involved in Brm-mediated chromatin remodeling (Chapter 3) and Wg signaling (Davidson et al., 2009). Considering the limited number of cellular kinases and the huge number of phosphorylation events in cells (de la Fuente van Bentem et al., 2008), I hypothesis that CycY may be involved in other signaling pathways by phosphorylating other substrates. I base this hypothesis in part on the fact that CycY is required for a broad range of developmental processes that appears to exceed the range of processes that requires Wg and Brm complex. This hypothesis could be tested in several ways. One could identify other CycY interaction partners by TAP purification and LC-MS/MS by using Drosophila cell lines or transgenic flies that stably express C-terminally TAP-tagged CycY. In addition, it would be very informative if one could identify *in vivo* CycY-dependant phosphorylation targets on a proteome-wide scale by quantitative MS (Smolka et al., 2005).

5.2.5 Implications for understanding human diseases

It is intriguing that CycY has been connected with several intestine-related human diseases. Recently, *CCNY* was identified as a potential susceptibility factor for inflammatory bowel disease (IBD), a complicated genetic disorder affecting the intestinal mucosa. A single nucleotide polymorphism (SNP) located in an intron of *CCNY* was found to be strongly associated with the two IBD subphenotypes, Crohn's disease and ulcerative colitis (Franke et al., 2008; Weersma et al., 2009). Another study found that human CycY is significantly upregulated in metastatic colorectal cancer cells (Ying-Tao et al., 2005). Davidson et al recently found that CycY binds to and activates Cdk14 to phosphorylate Wg/Wnt co-receptor LRP6/Arrow to activate Wg/Wnt signaling (Davidson et al., 2009). Interestingly, adenomatous polyposis coli (APC), a component of Wnt signaling pathway, is a tumor suppressor gene, which encodes a cytoplasmic protein that can bind to and promote the degradation of β-catenin. APC mutation is a common initiating factor in most human colorectal tumors (Kinzler and Vogelstein, 1996). Although CycY has been implicated in these human diseases, it is still unclear how CycY is involved. The generation of *CycY* null and tissue-specific knockdown animal models described in this dissertation provides valuable tools that may help us to further understand CycY's cellular functions and potential roles in these human diseases. For example, we know that CycY is highly conserved between Drosophila and human, but we do not know whether they are functionally interchangeable. To test this, one could generate transgenic flies that harbor human CCNY to see whether $CycY^{E8}$ null mutant phenotypes can be rescued. One SNP has been identified in an intron of *CCNY* in patients with IBD. However, the molecular consequence of this mutation has not been determined. Does this have any impact on the transcription or translation of *CCNY*?

Once this has been determined, further testing the behavior of *CycY* null mutant flies with *CCNY* mutant transgenes may shed some light on the mechanism of the inflammatory bowel disease.

This study initiated an interesting discussion about the function of a novel conserved cyclin, CycY. The more I read, work, write, and think about it, the stronger I feel that there is much to be elucidated about *CycY* functions in the future.

APPENDICES

Appendix A. Fly strains used in the study described in Chapter 2.

Finely lab #	Stock center & numbers	Alias or genotypes	References
352	Bloomington 11374	*crol*^{04418}	(D'Avino and Thummel, 1998)
286	Bloomington 4513	*Eip63E*^{81}	(Stowers et al., 2000)
9	Bloomington 3687	*Df(3L)GN50 (Eip63E*^{GN50}*)*	(Stowers et al., 2000)
232	Bloomington 7574	*Df(3L)Exel6095 (Eip63E*^{Df6095}*)*	-
606	Bloomington 7575	*Df(3L)Exel6096 (Eip63E*^{Df6096}*)*	-
103	Bloomington 3664	P{*Δ2-3*}	(Robertson et al., 1988)
422	Bloomington 2121	*ovo*^{D1} *neoFRT40A*	(Chou et al., 1993)
162	Bloomington 5138	*Tubulin-Gal4*	-
197	Bloomington 7513	*Df(2L)Exel6030 (CycY*^{Df6030}*)*	this study
208	Harvard Exelixis d03228	*d03228*	this study
355	Bloomington 8403	*FRT40A*	-
358	Bloomington 1929	*hs-FLP*	-
12	Bloomington 4533	*w; Sco/CyO Act5C-GFP*	-
41	-	*w; CyO/Sp; TM3 Ser/Sb*	VanBerkum's lab
692	-	*w; CycY*^{E8}*/CyO; TM3 Ser/Sb*	this study
693	-	*w; CycY*^{E8}*/CyO, Act5C-GFP*	this study
702	-	*w; CycY*^{E8} *FRT40A/CyO*	this study

Appendix B. Plasmids used or constructed in the study described in Chapter 2.

Plasmids Finely lab #	Plasmids Alias	Source or References
289	pCaspeR2	(Thummel et al., 1988)
977	pCaspeR2-CycY	This study
339	pCaspeR-hs	(Thummel et al., 1988)
1006	pCaspeR-hs-CycY	This study
966	pMT-Gal4	(Klueg et al., 2002)
812	pAS1	(A. Soans, and R.L.F., unpublished)
897	pAS1-CycY	This study
986	pAS1-CycY S389A	This study
985	pAS1-CycY S388/389A	This study
991	pAS1-Cyc39 S389E	This study
1000	pAS1-Cyc39 S388E	This study
961	pAS1-Eip63E	This study
1016	pAS1-Eip63E (B1, G243A)	This study
903	pAS1-Eip63E(B1, K234M)	This study
893	pAS1-β-tubulin	This study
904	pAS1-GFP	This study
894	pAS1-Cdk2	This study
895	pAS1-Cdk4	This study
896	pAS1-Cdk5	This study
898	pAS1-koko	This study
899	pAS1-CycD	This study
900	pAS1-CycE	This study
901	pAS1-CycK	This study
902	pAS1-Dap	This study
905	pAS1-p35	This study
841	pUAST-NTAP	(Veraksa et al., 2005)
844	pDL4	This study
928	pDL4-CycY	This study
983	pDL4-CycY S388/389A	This study
984	pDL4-CycY S389A	This study
992	pDL4-CycY S389E	This study
999	pDL4-CycY S388E	This study

933	pDL4-Eip63E	This study
934	pDL4-Eip63E G243A	This study
936	pDL4-Eip63E I249L	This study
937	pDL4-Eip63E (B1 K234M)	This study
924	pDL4-Cdc2rk	This study
925	pDL4-Cdk2	This study
926	pDL4-Cdk4	This study
927	pDL4-Cdk5	S. Guest and H. Zhang
923	pDL4-β-tubulin	This study
938	pDL4-GFP	This study
931	pDL4-CycK	This study
930	pDL4-CycD	This study
929	pDL4-koko	This study
932	pDL4-Dap	This study
939	pDL4-p35	This study
910	pDL2	This study
914	pDL2-CycY	This study
919	pDL2-Eip63E (A1)	This study
911	pDL2-Cdc2rk	This study
912	pDL2-Cdk2	This study
913	pDL2-Cdk4	This study
915	pDL2-Koko	This study
916	pDL2-Cyclin D	This study
917	pDL2-Cyclin J	This study
918	pDL2-Cyclin K	This study
920	pDL2-Side	This study
921	pDL2-tinman	This study
922	pDL2-tintin	This study
969	pCeMM-NTAP (GS)	(Burckstummer et al., 2006)
975	pDL5	This study
979	pDL5-CycY	This study
980	pDL5-Snr1	This study

Appendix C. Transgenic fly strains generated for the study described in Chapter 2.

Finley lab #	Alias	Genotypes	Plasmids for P-element transformation	Used in this study?
636	2T1	*UAS-CycY*	pAS1-CycY	No
637	2T11	*w; UAS-CycY/CyO; TM3 Ser/Sb*	pAS1-CycY	No
638	2T35	*w; CyO/Sp; UAS-CycY/TM3 Ser*	pAS1-CycY	No
639	2T44	*w; CyO/Sp; UAS-CycY/TM3 Ser*	pAS1-CycY	Yes
640	2T46	*w; CyO/Sp; UAS-CycY/TM3 Ser*	pAS1-CycY	No
642	2T44H	*w; CyO/Sp; UAS-CycY/UAS-CycY*	pAS1-CycY	Yes
643	2T46H	*w; CyO/Sp; UAS-CycY/UAS-CycY*	pAS1-CycY	No
655	3T1	*P{CycY}; CyO/Sp; TM3 Ser/Sb*	pCaspeR2-CycY	No
656	3T2	*P{CycY}; CyO/Sp; TM3 Ser/Sb*	pCaspeR2-CycY	No
657	3T11	*w; P{CycY}/CyO; TM3 Ser/Sb*	pCaspeR2-CycY	No
658	3T12	*w; P{CycY}/CyO; TM3 Ser/Sb*	pCaspeR2-CycY	No
659	3T13	*w; P{CycY}/CyO; TM3 Ser/Sb*	pCaspeR2-CycY	No
660	3T21	*w; CyO/Sp; P{CycY}/TM3 Ser*	pCaspeR2-CycY	Yes
661	3T22	*w; CyO/Sp; P{CycY}/TM3 Ser*	pCaspeR2-CycY	No
662	3T27	*w; CyO/Sp; P{CycY}/TM3 Ser*	pCaspeR2-CycY	Yes
663	4T1	*hs-CyY; CyO/Sp; TM3 Ser/Sb*	pCaspeR-hs-CycY	No
664	4T11	*w; hs-CycY/CyO; TM3 Ser/Sb*	pCaspeR-hs-CycY	No
665	4T12	*w; hs-CycY/CyO; TM3 Ser/Sb*	pCaspeR-hs-CycY	No
666	4T13	*w; hs-CycY/CyO; TM3 Ser/Sb*	pCaspeR-hs-CycY	No
667	4T21	*w; CyO/Sp; hs-CycY/TM3 Ser*	pCaspeR-hs-CycY	No
668	4T22 (G6.1)	*w; CyO/Sp; hs-CycY/TM3 Ser*	pCaspeR-hs-CycY	Yes
669	4T23	*w; CyO/Sp; hs-CycY/TM3 Ser*	pCaspeR-hs-CycY	No
699	E8;G6.1/G6.1	*w; $CycY^{E8}$/CyO; hs-CycY/hs-CycY*	pCaspeR-hs-CycY	Yes
700	E8;G6.1/Ser	*w; $CycY^{E8}$/CyO; hs-CycY/TM3 Ser*	pCaspeR-hs-CycY	Yes
670	5T1-1	*UAS-Eip63E; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E	No
671	5T1-2	*UAS-Eip63E; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E	No
672	5T11	*w; UAS-Eip63E/CyO; TM3 Ser/Sb*	pAS1-Eip63E	No
673	5T12	*w; UAS-Eip63E/CyO; TM3 Ser/Sb*	pAS1-Eip63E	No
674	5T21	*w; CyO/Sp; UAS-Eip63E/TM3 Ser*	pAS1-Eip63E	No
675	5T22	*w; CyO/Sp; UAS-Eip63E/TM3 Ser*	pAS1-Eip63E	No
676	5T23	*w; CyO/Sp; UAS-Eip63E/TM3 Ser*	pAS1-Eip63E	No
677	5T24	*w; CyO/Sp; UAS-Eip63E/TM3 Ser*	pAS1-Eip63E	No

678	6T1	*UAS-Eip63E G243A; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
679	6T2	*UAS-Eip63E G243A; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
680	6T3	*UAS-Eip63E G243A; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
681	6T4	*UAS-Eip63E G243A; CyO/Sp; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
682	6T11	*w; CyO/UAS-Eip63E G243A; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
683	6T12	*w; CyO/UAS-Eip63E G243A; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
684	6T13	*w; CyO/UAS-Eip63E G243A; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
685	6T14	*w; CyO/UAS-Eip63E G243A; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
686	6T15	*w; CyO/UAS-Eip63E G243A; TM3 Ser/Sb*	pAS1-Eip63E (G243A)	No
687	6T21	*w; CyO/Sp; UAS-Eip63E G243A/TM3 Ser*	pAS1-Eip63E (G243A)	No
688	6T22	*w; CyO/Sp; UAS-Eip63E G243A/TM3 Ser*	pAS1-Eip63E (G243A)	No
689	6T23	*w; CyO/Sp; UAS-Eip63E G243A/TM3 Ser*	pAS1-Eip63E (G243A)	No
690	6T24	*w; CyO/Sp; UAS-Eip63E G243A/TM3 Ser*	pAS1-Eip63E (G243A)	No
691	6T25	*w; CyO/Sp; UAS-Eip63E G243A/TM3 Ser*	pAS1-Eip63E (G243A)	No

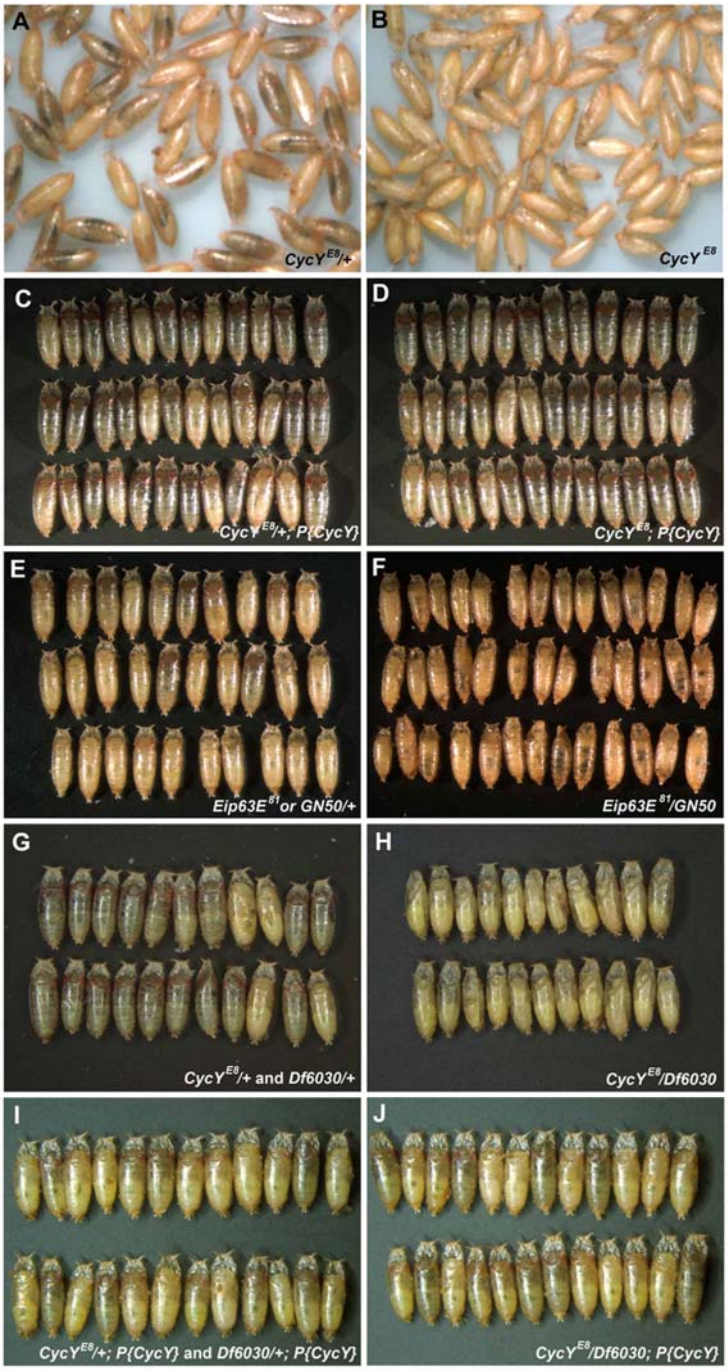
Appendix D. Primer pairs used to characterize the deletion after P-element mobilization.

Primer pairs	Region to be amplified
DL20/DL17	Span the whole P-element inserted region
DL44/DL17	The right end of the P-element
DL45/DL20	The left end of the P-element
DL83/DL17	*CycY* gene region
DL78/DL58	*CycY* gene region
DL77/DL58	*CycY* gene region
DL77/DL21	*CycY* gene region
DL78/DL17	*CycY* gene region
DL83/DL57	*CycY* gene region
DL72/DL84	Crol gene region
DL74/DL82	Crol gene region
DL56/DL81	Crol gene region
DL73/DL80	*Crol* gene region
DL55/DL79	*Crol* gene region
DL96/DL97	*Crol* gene region
DL96/DL98	*Crol* gene region
DL98/DL106	*Crol* gene region
DL107/DL106	*Crol* gene region
DL107/DL104	*Crol* gene region
DL93/DL95	*Pde1c* gene region
DL94/DL95	*Pde1c* gene region
DL75/DL59	*Pde1c* gene region
DL72/DL57	*Crol* and *CycY* gene region
DL72/DL58	*Crol* and *CycY* gene region
DL20/DL58	*Crol* and *CycY* gene region
DL99/DL101	Intergene between *CycY* and *Pde1c*
DL100/DL103	Intergene between *CycY* and *Pde1c*
DL76/DL21	Intergene between *CycY* and *Pde1c*
DL72/DL59	*Crol*, *CycY*, and *Pde1c* gene region
DL20/DL59	*Crol*, *CycY*, and *Pde1c* gene region
DL98/DL99	*Crol*, *CycY*, and *Pde1c* gene region
DL94/DL98	*Crol*, *CycY*, and *Pde1c* gene region

Appendix E. Sequences of primers used in the study described in Chapter 2.

Primers Finley lab #	Primers Alias	Sequences
469	DL17	5’ TACTCCCGGTGGCAATAG
648	DL20	5' GTGCGATTGCGTTGTTCTTA
649	DL21	5' CGTAGGGAAATTCGAGGTGG
664	DL42	5' CAAGGCGGTTTATCTGATCG
522	DL43	5' AATGATTCGCAGTGGAAGGCT
523	DL44	5' CACCCAAGGCTCTGCTCCCACAAT
524	DL45	5' CGACACTCAGAATACTATTCC
525	DL46	5' AATTTGCGAGTACGCAAAGC
669	DL55	5’ TTTGCCTTACATTGTCTCTC
670	DL56	5’ ATGACAAGGGACACGAAAAT
671	DL57	5’ CCAATTGCCAAACGAAAGAT
672	DL58	5’ ACCCAATAATCCCGATTTGG
673	DL59	5' AGAGGCATTGCAGGATATG
686	DL72	5' CATCCCATCATATCCGACC
687	DL73	5' GTAACACGAATCCCCTAACC
688	DL74	5’ CCGCTGCTAATTGATGATTG
689	DL75	5’ CTCGGCATCTGAAAACAGG
690	DL76	5’ GCTTTGAGCGTTCAAGTTTG
691	DL77	5’ GAGCACGACTCTAACTTCTTC
692	DL78	5’ CCAGGTAGACTAGCGTGATG
693	DL79	5’ CCTGTACTCGCTTGTCTCTC
694	DL80	5’ GATCATTCTTGTTTCTGGACC
695	DL81	5’ AAACCAGGACCTATGCAAAC

696	DL82	5’ GGATCTGGACACAAGAATGC
697	DL83	5’ AATCGATTTGTGCCTGAAGC
698	DL84	5’ CCGCGTCTCATTCAGTTTTC
707	DL93	5’ TCACCTGTTTTCAGATGCCGAG
708	DL94	5’ GGGCCAAGCACAAATACAAACG
709	DL95	5’ GATCGACGCGTCTGTCGTTCC
710	DL96	5’ AATCGCACGCACACACACACATG
711	DL97	5’ ACAAGGCGGTTTATCTGATCGG
712	DL98	5’ TGGTGAACGGCGAACAGAGC
713	DL99	5’ TCAAGAAAGGAACGACAGACG
808	RA19	5’ TACTATTCCTTTCACTCGCACTTATTG
714	DL100	5’ GGGACAAAAGTGAGAGCAG
715	DL101	5’ CTGCTCTCACTTTTGTCCC
716	DL102	5’ GCGGATGTCTACTAGTAGCC
717	DL103	5’ GGCTACTAGTAGACATCCGC
718	DL104	5’ GTAATTGGAGTAAGTGCAGGTG
719	DL105	5’ CACCTGCACTTACTCCAATTAC
720	DL106	5’ CGATCAGATAAACCGCCTTG
721	DL107	5’ TTGAACTTCCTAAGTGTGGC
722	DL108	5’ AGGAGAATGGCACCCAAC
891	5’ RT-F	5' TTGACTGTATCGCCGGAATTC
892	3’ RT-R	5' CCGGAATTAGCTTGGCTGCAG



Appendix F. *CycY* and *Eip63E* null mutant pupae are smaller than wild-type pupae. A collection of 30 to 40 pupae of $CycY^{E8}$/+ (A), $CycY^{E8}$ (B), $CycY^{E8}$/+; *P{CycY}* (C), $CycY^{E8}$; *P{CycY}* (D), $Eip63E^{GN50}$/+ and $Eip63E^{81}$/+ (E), $Eip63E^{GN50}/Eip63E^{81}$ (F), $CycY^{E8}$/+ and *Df(2L)Exel6030*/+ (G), $CycY^{E8}$/*Df(2L)Exel6030* (H), $CycY^{E8}$/+; *P{CycY}* and *Df(2L)Exel6030*/+; *P{CycY}* (I), $CycY^{E8}$/*Df(2L)Exel6030; P{CycY}* (J) were photographed and the relative pupal length on the image was measured. Images A-F were taken at the same magnification and images G-J were taken at the same magnification.

Appendix G. Fly strains used in the study described in chapter 3

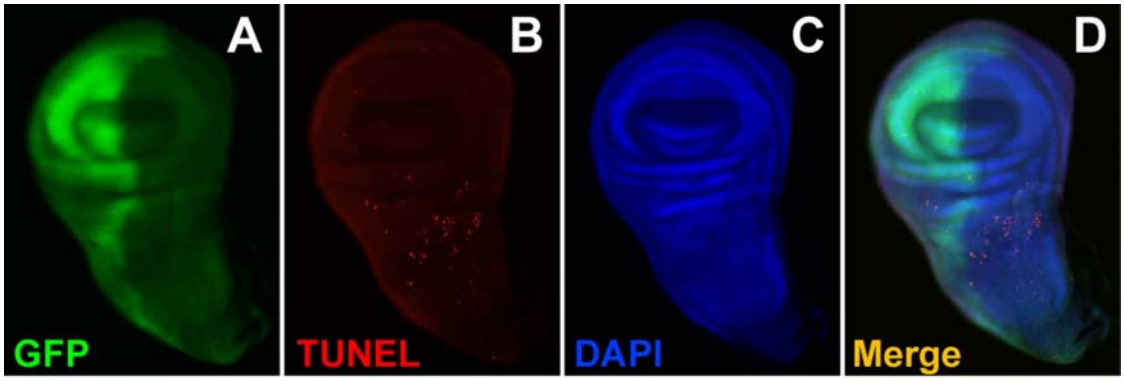
Finely lab #	Stock center & numbers	Alias or genotypes	References
210	Bloomington 1104	*GMR-Gal4*	(Freeman, 1996)
284	Bloomington 6356	*en-Gal4*	(Brand and Perrimon, 1993; Sigrist and Lehner, 1997)
446	Bloomington 1774	*69B-Gal4*	(Brand and Perrimon, 1993)
161	Bloomington 1973	*e22c-Gal4*	(Zraly et al., 2003)
290	Bloomington 4414	*Act5C-Gal4*	-
424	-	*UAS-Snr1-2, $Snr1^{R3}$*	(Zraly et al., 2003)
448	-	*UAS-Brm^{K804R}*	(Elfring et al., 1998)
434	-	*$y^{1}w^{*}$ hs-FLP; Ubi-GFP FRT40A*	Dr. Dongbin Xu
407	VDRC 60008	*UAS-dicer2*	(Lee et al., 2004)
482	VDRC 12644	*UAS-Snr1i*	(Terriente-Felix and de Celis, 2009)
484	VDRC 37720	*UAS-Brmi*	(Terriente-Felix and de Celis, 2009)
692	-	*$CycY^{E8}$*	(Liu and Finley Jr, 2010)
702	-	*$CycY^{E8}$ FRT40A*	(Liu and Finley Jr, 2010)
642	-	*UAS-CycY*	(Liu and Finley Jr, 2010)
668	-	*hs-CycY*	(Liu and Finley Jr, 2010)

Appendix H. Plasmids used or constructed for the study described in chapter 3

Plasmids Finely lab #	Plasmids Alias	Source or References
955	pWIZ	(Lee and Carthew, 2003)
952	pWIZ-CycYi^{N}	This study
953	pWIZ-CycYi^{C}	This study
766	pTLJ03	(Parrish et al., 2004)
943	pTLJ03-Eip63E A1	This study
944	pTLJ03-Eip63E A1 I249L	This study
945	pTLJ03-Eip63E B1	This study
946	pTLJ03-Eip63E B1 I249L	This study
947	pTLJ03-Eip63E(B1, K234M)	This study
948	pTLJ03-Snr1	This study
949	pTLJ03-Snr1(T102/198A)	This study
950	pTLJ03-Snr1(T102A)	This study
978	pTLJ03-CycY	This study
981	pTLJ03-CycY S388/389A	This study
982	pTLJ03-CycY S389A	This study
990	pTLJ03-CycY S389E	This study
998	pTLJ03-CycY S388E	This study
378	pET-28a(+)	-
1011	pET28a-Eip63E	This study
1012	pET28a-Snr1	This study
1017	pET28a-CycY	This study
1029	pET28a-nCycY	This study
1007	pMal-c2	-
1009	pMalc2-Eip63E	This study
1010	pMalc2-Snr1	This study
1030	pMalc2-nCycY	This study
1033	pMalc2-Snr1 T102/198A	This study
1034	pMalc2-Snr1 T102A	This study
1035	pMalc2-Snr1 T198A	This study

Appendix I. Transgenic fly strains generated for the study described in chapter 3

Finley lab #	Alias	Genotypes	Plasmids for P-element transformation	Used in this study?
644	3R2	*CycYi*	pWIZ-CycYi^{C}	No
645	3R22	*w; CycYi^{C2}/CyO; Ser/Sb*	pWIZ-CycYi^{C}	Yes
646	3R22H	*w; CycYi^{C2}; Ser/Sb*	pWIZ-CycYi^{C}	Yes
647	3R23	*w; CycYi/CyO; Ser/Sb*	pWIZ-CycYi^{C}	No
648	3R51	*w; Sp/CyO; Ser/CycYi*	pWIZ-CycYi^{C}	No
649	3R52	*w; Sp/CyO; Ser/CycYi^{C1}*	pWIZ-CycYi^{C}	Yes
650	3R53	*w; Sp/CyO; Ser/CycYi*	pWIZ-CycYi^{C}	No
651	3R54	*w; Sp/CyO; Ser/CycYi*	pWIZ-CycYi^{C}	No
652	3R21B1	*w; CycYi/CyO Act5C-GFP; Ser/Sb*	pWIZ-CycYi^{C}	Yes
653	3R22B1	*w; CycYi/CyO Act5C-GFP; Ser/Sb*	pWIZ-CycYi^{C}	Yes
654	3R23B1	*w; CycYi/CyO Act5C-GFP; Ser/Sb*	pWIZ-CycYi^{C}	Yes
752	2R2	*CycYi*	pWIZ-CycYi^{N}	Yes
753	2R21	*w; CyO/CycYi^{N1}; Ser/Sb*	pWIZ-CycYi^{N}	Yes
754	2R22	*w; CyO/CycYi; Ser/Sb*	pWIZ-CycYi^{N}	Yes
755	2R51	*w; CyO/Sp; CycYi^{N2}/Ser*	pWIZ-CycYi^{N}	Yes
756	2R52	*w; CyO/Sp; CycYi/Ser*	pWIZ-CycYi^{N}	Yes



Appendix J. Wing imaginal discs upon knockdown of *CycY* do not show abnormal proportion of apoptotic cells. Wing imaginal discs (*en-Gal4/+; UAS-CycYi^{C1}/UAS-GFP*) were stained with GFP (Green, A), TUNEL (Red, B), and DAPI (Blue, C).

Appendix K. Fly strains used in the study described in Chapter 4

Finley lab #	Stock center & numbers	Alias or genotypes	References or sources
434	-	*$y^1 w^*$ hs-FLP; Ubi-GFP FRT40A*	Dr. Dongbin Xu
435	-	*$y^1 w^*$ ey-FLP; Ubi-GFP FRT40A*	Dr. Dongbin Xu
442	-	*$y^1 w^*$;Sp/CyO* [y+]*;TM2/TM6B Tb*	Dr. Lei Zhang
711	-	*$y^1 w^*$; CyO* [y+]*/Sp; TM6B Tb/Sb*	this study
704	-	*$y^1 w^*$; FRT40A/CyO* [y+]*; TM6B Tb/Sb*	this study
705	-	*$y^1 w^*$; $CycY^{E8}$/CyO* [y+]*; TM6B Tb/Sb*	this study
777	-	*$y^1 w^*$; $CycY^{E8}$/CyO* [y+]*; TM6B Tb/$Eip63E^{81}$*	this study
778	-	*$y^1 w^*$; $CycY^{Df6030}$/CyO* [y+]*; TM6B Tb/$Eip63E^{Df6096}$*	this study
779	-	*$y^1 w^*$; $CycY^{Df6030}$/CyO* [y+]*; TM6B Tb/$Eip63E^{Df6095}$*	this study
437	Bloomington 5885	*Sgs3-GFP*	(Biyasheva et al., 2001)
706	-	*$y^1 w^*$; CyO* [y+]*/Sp; sgs3-GFP/TM6B Tb*	this study
710	-	*$y^1 w^*$; $CycY^{E8}$/CyO* [y+]*; sgs3-GFP/TM6B Tb*	this study
535	-	*w^*; $Atg1^{\Delta 3D}$/TM6B*	Thomas P. Neufeld's lab
717	-	*$y^1 w^*$; $CycY^{E8}$/CyO* [y+]*; $Atg1^{\Delta 3D}$/TM6B Tb*	this study
534	-	*$y^1 w^*$; $TOR^{\Delta 6B}$/CyO*	Thomas P. Neufeld's lab
718	-	*$y^1 w^*$; $TOR^{\Delta 6B}$/CyO* [y+]*; $CycYi^{C1}$/TM6B Tb*	this study (also see Chapter 3 for details bout *$CycYi^{C1}$*)
719	-	*$y^1 w^*$; $TOR^{\Delta 6B}$/CyO* [y+]*; Tub-Gal4/TM6B Tb*	this study

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ABSTRACT

DETERMINATION OF THE ESSENTIAL FUNCTIONS OF A CONSERVED CYCLIN, CYCLIN Y, IN DROSOPHILA

by

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The Drosophila gene *CG14939* encodes a member of a highly conserved family of cyclins, the Y type cyclins, which have not been functionally characterized in any organism. Here I report the generation and phenotypic characterization of a null mutant of *CG14939*, which we rename *Cyclin Y* (*CycY*). I show that the null mutant, $CycY^{E8}$, is homozygous lethal with most mutant animals arresting during pupal development. The mutant exhibits delayed larval growth and major developmental defects during metamorphosis. Heat shock-induced expression of *CycY* at different times during development resulted in variable levels of rescue, the timing of which suggests a key function for zygotic *CycY* during the transition from third instar larvae to prepupae. *CycY* also plays an essential role during embryogenesis since zygotic null embryos from null mothers fail to hatch into first instar larvae. Furthermore I show that *CycY* is required for adult viability, especially in males. I provide evidence that the *CycY* protein (CycY) interacts with Eip63E, a Cyclin-dependent kinase (Cdk) for which no cyclin partner had previously been identified. Like *CycY*, the *Eip63E* gene has essential functions during

embryogenesis, larval development, and metamorphosis. My data suggest that CycY/Eip63E form a cyclin/Cdk complex that is essential for several developmental processes.

To gain insight into the cellular functions of CycY and to identify signaling pathways to which it belongs, I used RNA interference (RNAi) to knock down *CycY* expression in specific tissues. I show that *CycY* is required for wing growth and wing vein development. I also show that *CycY* genetically interacts with *Snr1* and *Brm*, two components of the Brm ATP-dependent chromatin remodeling complex, and that CycY can physically interact with Snr1. Furthermore, I show that downstream targets of the Brm complex are misregulated in *CycY* mutants. Taken together, these data suggest that *CycY* may be involved in gene regulation by modulating Brm complex activity.

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